CONTROL OF CELLULAR DNA REPLICATION

IN ADENOVIRUS INFECTED CELLS

by

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STATEMENT

Flow cytometry experiments done in Sydney (see Chapter 4) were part of a collaborative project with Drs. Ian Taylor and Jim Murray. The cyclic nucleotide phosphodiesterase assay (Chapter 5) was done in collaboration with Dr. Mike Appleman. The rest of the work in this thesis is my own.

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ABBREVIATIONS

Ad	adenovirus
5'-AMP	adenosine 5'-monophosphate
ATPase	adenosine triphosphatase
CsCl	cesium chloride
^{o}C	degrees celsius
Ci	curie
mCi	millicurie
µCi	microcurie
cm	centimeter
nm	nanometer
c.p.m.	counts per minute (of radioactivity)
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid (disodium salt)
g	gram weight
mg	milligram
l	liter
ml	milliliter
µl	microliter
M	molar (moles/liter)
mM	millimolar
µM	micromolar
mmole	millimole
N	normal
n	haploid chromosome number
2n	diploid " "
4n	G_2 diploid " "

RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
r.p.m.	revolutions per minute
poly A	polyadenylated
ρ	density (g/ml)
S	sedimentation coefficient
SDS	sodium dodecyl sulphate
Tris	tris (hydroxymethyl) aminomethane (Trizma base)
w/v	weight (g)/volume (100ml)
v/v	volume (ml)/volume (100ml)

ABSTRACT

It is commonly argued that oncogenic transformation of cells results from alterations to the controls of normal cellular proliferation. The experiments reported in this thesis investigate this hypothesis using the human adenovirus type 5 (Ad 5), which has been shown to transform both human and rodent cells in vitro. Some of these cells also cause tumors when inoculated into syngeneic animals. The experiments provide evidence that Ad 5 can induce rodent cells into a cell cycle under different conditions--which prevent normal cell cycling, including some conditions which are used to select transformed cells. Other experiments show that the transcriptional unit of the Ad 5 genome responsible for inducing alterations to the rodent cell cycle is the same as that required to initiate transformation. Such results are discussed throughout the thesis in terms of transformation mechanisms, and provide confirmatory evidence for the above hypothesis that transformation occurs by alterations to cellular growth controls.


FACE IT HARRY! CANCER
IS A GROWTH INDUSTRY

"To travel hopefully is a better thing

than to arrive ....."

R.L. Stevenson

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CHAPTER 1

VIRUSES, CANCER, AND CELLULAR PROLIFERATION: A REVIEW

1.1 PREAMBLE

> "We shall not cease from exploration
> And the end of all our exploring
> Will be to arrive where we started
> And know the place for the first time"

This quotation from "The Four Quartets" by T.S. Eliot seems to be a particularly apt commentary on the state of scientific research into the origins of human cancer. For although the causes of human cancer have been a major focal point for biomedical research for many years, we still do not understand the molecular basis of cancer, but our perspectives on the origins of cancer keep changing. One area of cancer research for which there is considerable evidence concerns the role viruses play in the etiology of cancer. Some people argue strongly that viruses play an important role in the induction of cancers under natural conditions (e.g. Todaro (1980); see Section 1.2), and there is in fact good circumstantial evidence to support this view, including evidence in man. Viruses for which the best evidence exists are the RNA tumor viruses and amongst the DNA tumor viruses, Epstein-Barr virus (EBV). In this latter case there is strong evidence of an association between EBV and Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) in humans, at least for some geographical areas (see Section 1.2.2).

A popular hypothesis concerning the way viruses cause cancer is one which argues that viruses express certain genes which directly alter cells in ways which render them neoplastic. This has led to

the concept of specific virus coded "tumor" or "transforming genes" which are both necessary and sufficient to change normal cells into neoplastic cells. Simian Virus 40 (SV 40), the adenoviruses, and Rous sarcoma virus (RSV; one of the RNA tumor viruses) are all well-characterised examples of viruses which have transforming genes. At least for the DNA tumor viruses, where the evidence is much less clear, whether or not such genes are sufficient for carcinogenesis is a matter of some debate. Some of the evidence for virus specific genes and transformation and cancer is discussed in following sections and in Chapter 4 (see introduction and discussion).

The concept of transforming genes being required to be expressed continuously to induce and maintain cancers has tended to dominate scientific research for several years. However, this view is a little restrictive, and it is not by any means the only mechanism that would enable a virus to cause cancer.

Several DNA viruses have been shown to cause specific cellular chromosome lesions both during transformation and lytic infections. For example, EBV-derived lymphoma cell lines have been shown to contain various chromosome abnormalities, the most common of which is a specific translocation between the human chromosomes 8 and 14 (see review by Klein, 1975). (Although similar chromosome lesions have been found in non-Burkitt's lymphomas (Zech et al., 1976) allowing the possibility that EBV is not directly responsible for the lesion.) Other chromosome lesions have also been shown for herpes simplex virus (HSV), SV 40, and

the adenoviruses (see Chapter 4). If such a mutation is selected, it might contribute considerably to the carcinogenic potential of a virus infected or transformed cell. At the extreme the viral transforming genes might only be required to induce a critical chromosome lesion which in itself is sufficient to cause cancer. Perhaps more realistically, as with two-stage chemical carcinogenesis (Berenblum, 1941), two or more separate processes might contribute to carcinogenesis by viruses. These could be the induction of specific chromosome lesions and the alteration of cellular growth controls by continuously expressed viral genes located on integrated DNA fragments. A model similar to this has recently been proposed by Bissell et al.,(1979) for the action of the transforming (src) gene of RSV.

Risk factor, as used by epidemologists, describes the role played by some factor (such as a virus) in increasing the probability with which diseases occur. Thus it is a "correlative" rather than a mechanistic term; it provides no explanation as to how a given agent acts to induce a disease, but rather a means of assessing the contributions of different factors in the etiology of a disease. This concept allows one to account for many of the observations which are inconsistent with continuous viral gene expression (see above) being both necessary and sufficient for carcinogenesis. Thus viruses may contribute to the probability of a cell becoming malignant without themselves necessarily being solely responsible for a particular cancer. In this context, the study of the ways in which viruses modify cellular growth control mechanisms might be useful in understanding what the risk factor for a given virus might be.

The experiments presented in this thesis are fundamentally concerned with alterations that occur when adenoviruses infect cells, particularly alterations to the regulatory controls of cellular DNA replication after adenovirus infection, and the role these might play in cellular transformation. These studies have connections with 3 main areas of cell and molecular biology which I have reviewed in this first chapter, in order to establish a context for the research work described later in the thesis. These areas are: (1) Tumor viruses and cancer; (2) Controls of cellular proliferation and eukaryote DNA synthesis; and (3) The molecular biology of the human adenoviruses. Other areas more directly concerned with the experimental work are reviewed in the introductions to Chapters 3 to 6.

1.2 THE ONCOGENIC VIRUSES AND CANCER

1.2.1 INTRODUCTORY REMARKS

Many types of DNA and RNA viruses have been shown to transform cells in tissue culture. Such virus transformed cells resemble tumor cells in a number of in vitro characteristics. However, not all such transformed cells form tumors when inoculated into susceptible animals, and in many cases it has been difficult to demonstrate that oncogenic viruses directly cause the formation of tumors in experimental animals. For humans, it is of course impossible to show directly that viruses cause cancers without the use of human subjects as experimental systems. In the absence of such a system, there are two alternative approaches to studying the etiological role of viruses in human cancers. One approach is to use classical epidemiological methods to find associations between viral infections and cancer as has been done profitably

for EBV and HSV (see Section 1.2.2) for BL and cervical carcinoma respectively. The other approach is to screen tumors and normal tissues for the presence of virus specific proteins or nucleic acid sequences which in the case of DNA fragments may be integrated into the cellular DNA. The presence or absence of such virus components is then correlated with tumor incidence. In the following sections selected examples of tumor viruses which are thought to play an etiological role in animal and human cancers are discussed.

1.2.2 HERPESVIRUSES

Evidence from studies with experimental animals, using animal herpesviruses has provided strong evidence for a causal link between herpesvirus infections and lymphoproliferative malignancies. For example, infection of marmosets or owl monkeys with simian herpesviruses (*Herpesvirus* saimiri and *Herpesvirus* ateles) causes the formation of lymphomas or lymphoblastic leukemias, which in most cases progress rapidly and kill the animals within 20 to 150 days (see review by Deinhardt and Deinhardt, 1979). Marek's disease in chickens (also known as neurolymphomatosis) is a lymphoproliferative malignancy which has been shown to be caused by a herpesvirus showing many similarities with EBV. However, there are some reports that the disease may result from an interaction between herpesvirus and endogenous type C RNA viruses (Deinhardt and Deinhardt, 1979). In man, the causal connection between herpesvirus infections and carcinogenesis is not so clearly established, but serological and epidemiological studies have provided a strong basis for suggesting that human herpesviruses do play an etiological role in human cancers.

In a series of detailed experiments by Hollinshead et al.,(1976), 90% of sera from 244 patients with squamous carcinomas of the cervix had antibodies to HSV-tumor-associated antigens compared to 11% from patients with nonsquamous cancers and 4% from normal controls, thus providing clear evidence of an association between HSV and cervical carcinoma. Studies such as this one appear to be representative of several sero-epidemiological investigations implicating HSV as a causal agent of human genital tumors (see Rawls et al., 1976; Nahmias et al., 1974). In a recent review by Aurelian et al.,(1980), several lines of evidence are summarised suggesting HSV type 2 is a causal agent of cervix cancer. It would appear that women infected with HSV type 2 are at significantly higher risk of developing cervical cancer than uninfected women, and generally HSV type 2 infection precedes the onset of neoplasia. HSV specific "early" antigens (see Section 1.4.1 for definition of "early" antigens) are invariably detected in patients with cervix cancer, the most common of which are antigens AG-4 and VP 134. In some cervical atypical cells viral mRNA corresponding to one or more of these early antigens has been detected (Jones et al., 1978). These antigens and viral mRNAs are not detected in normal cervical cells.

Nucleic acid hybridisation of thin frozen sections of cervical tumors indicated that there was an association between tumor tissues and expression of HSV type 2 RNA sequences (Maitland et al., 1981). Of cervical biopsies with abnormal cytology, 67% bound HSV type 2 [^{3}H] labeled DNA probes whereas only 23% of normal biopsies bound labeled probes. In addition, 7% of labeled

phage lambda DNA bound to abnormal cervical biopsies. These figures represent averages of all their experiments, but in some particular experiments all 3 DNA probes bound to tissue biopsies from carcinomas. The binding of HSV type 2 DNA sequences to normal tissues suggests at least that the correlation between HSV sequences and carcinogenesis is not a strict one, and the "apparent nonspecific" binding of phage lambda complicates the interpretation. However, the higher binding to RNA from cancer cells does suggest a higher level of HSV expression in some of these cells which might be related to oncogenesis. Exact interpretation would seem to be impossible on the basis of current evidence. This lack of a clear and universal association between human cancer and integrated or free viral DNA has led to the suggestion that HSV might act as an initiating agent, and only in the presence of other factors as well, do human genital cancers develop (zur Hausen, 1980).

Perhaps the best candidate of the DNA viruses as an etiological agent for human cancers is EBV. EBV infects human B lymphocytes and appears to be responsible for initiating the formation of continuous lymphoblastoid cell lines (Pope et al., 1968; Epstein and Achong, 1977). EBV has been reported to be strongly associated with two human malignant tumors, BL originally discovered in Uganda by Dennis Burkitt in the 1950s and NPC. EBV also causes infectious mononucleosis (see Henle et al., 1979).

EBV was originally isolated by Epstein and colleagues (see Epstein et al., 1964) from biopsies taken from an African child with BL and subsequently many patients with BL have been shown to be seropositive for EBV antigens (see review by Henle et al., 1979). Not only did BL patients from Africa show antibodies to EBV but so did healthy children from the same region, and children from many parts of the world (see reviews by Epstein and Achong, 1977; and Henle et al., 1979). However, antibody titres were generally 8 to 10 times higher in BL and NPC patients than in healthy controls. NPC is a malignant tumor that arises in the epithelial lining of the space behind the nose. This tumor occurs in high frequency in Southern China, Tunisia, and East Africa but is rare elsewhere in the world (see Henle et al., 1979).

Whereas the evidence linking EBV to infectious mononucleosis is ~~unconditionally~~ unequivocal (see Epstein and Achong, 1977), the involvement of the virus in the two cancers (BL and NPC) is less clearcut. The case for a causal relation between EBV and BL rests on the following types of evidence. Firstly, the detection of viral DNA or viral antigens in the tumor. For example, in more than 95% of histologically typical tumors derived from BL endemic areas (e.g. Africa and New Guinea), EBV DNA sequences have been detected (see zur Hausen, 1980) which in many cases (>90%) is correlated with the expression of an EBV-specified nuclear antigen (Reedman and Klein, 1973). Mostly the viral DNA is recovered as free copies but in some cases it has been shown to be integrated into the host chromosome (Adams and Lindahl, 1975). Two other lines of evidence would also appear

to be important in providing a causal association with EBV and cancer. These are the higher EBV specific antibody titres in lymphoma patients compared with controls (see above) and the correlation between antibody patterns and tumor prognosis. For example, in an extensive sero-epidemiological study undertaken in Uganda involving more than 40,000 children, de-Thé and colleagues (de-Thé et al., 1978) showed that children with high antibody titres to EBV were at high risk of developing BL. However, this study indicated that the oncogenic potential of EBV was only realised in exceptional circumstances and that other factors were clearly required for EBV to cause malignant development.

Other important evidence for EBV in human cancer comes from experiments with animals and cell culture studies. Shope et al., (1973) showed that infection of cottontop marmosets with EBV or inoculation with autologous in vitro EBV-transformed cells resulted in neoplasia resembling human malignant lymphoma. No such tumors developed in uninoculated animals. Such results suggest that EBV is capable of inducing a malignant lymphoma in primates thus supporting an argument for EBV as a causal agent in human lymphomas. In vitro studies in which normal peripheral leukocytes from female infants were cocultured with lethally irradiated cells from male BL patients containing herpes-type virus, showed that the BL cells stimulated proliferation of the normal leukocytes (Henle et al., 1967), but neither cell type grew when cultured separately. In all female leukocytes

stimulated to proliferate herpes-type viral antigens were detectable as well as a C-group chromosal marker typical of BL cells. A similar type of lymphoproliferative transformation of human leukocytes by EBV has been reported by others (e.g. Pope et al., 1968).

All five lines of evidence discussed above have provided considerable support for a causal relation between EBV and BL, although there are still many puzzling observations. A similar set of associations linking EBV to NPC has also been shown in many studies, the details of which are discussed in the reviews by Epstein and Achong (1977) and Henle et al., (1979).

The distribution of BL in the world is interestingly closely related to the distribution of malaria. For example, there are very few reported cases of BL in Africa which fall outside areas in which malaria is endemic (see review by Henle et al., 1979), indeed it was just this distribution which led Burkitt to suggest that BL was caused by a mosquito-transmitted agent (Burkitt, 1958). A similar association between BL and malaria has been observed in New Guinea (Epstein and Achong, 1977). Outside of Africa and New Guinea, sporadic cases of BL have been reported including the United States, and in many such cases there is a limited association between EBV and BL. For example, Andersson et al.,(1976)showed that only 2/12 American patients diagnosed as having BL, and having no history of malarial infections, had any positive association with labeled EBV specific complementary RNA by nucleic acid hybridisation.

A similar lack of association between BL and EBV nucleic acids was demonstrated by Bornkamm et al., (1976) for European patients.

Results from these kinds of experiments suggest that BL, and also NPC (see below), probably have multicausal origins. That is, EBV might be required to modify cells (such as by altering growth properties or causing chromosal abnormalities, see Henle et al., 1967, and the review by Klein,1975) in a way which makes them more susceptible to developing into a lymphoma. For the African lymphoma, the concomitant presence of malaria may act to stimulate proliferation of EBV infected B cells by causing some form of immunosuppression. Specific and nonspecific (anti-sheep red blood cell response) immune suppression have been demonstrated by infection of animals with two strains of murine malaria, which for specific suppression appeared to be mediated by macrophages (Lelchuk and Playfair, 1980). A similar phenomenon has been reported in primates after infection with the human falciparum malaria (Taylor and Siddiqui, 1978).

For NPC, the evidence implicating EBV as the causal agent is perhaps even stronger than for BL as all biopsies of NPC obtained anywhere in the world have so far revealed the presence of EBV DNA, which is lacking in all other carcinomas of the head and neck (Desgranges et al., 1975; reviews by Epstein and Achong (1977) and Henle et al.(1979)). Nevertheless not everyone who is infected with EBV develops NPC or BL. For NPC there appears to be a clear genetic predisposition to the disease as Chinese patients with the carcinoma for example also show a higher

frequency of a certain histocompatibility (HLA) antigen (see Henle et al., 1979). In addition, the long interval between the time of primary EBV infection and the onset of this tumor suggests that cofactors are required in order to cause cells to become malignant (see Epstein and Achong, 1977).

Summary

The data discussed above have provided strong and extensive circumstantial evidence for a causal link between herpesvirus infections and some human tumors. However, the association with malaria for BL, the genetic factors for NPC, and the lack of a clearcut association of these tumors with EBV in Western countries, provides evidence for multicausal origins of these tumors. These data are consistent with a model suggesting that herpesviruses increase the "risk" with which cancers occur, but are not in themselves sufficient to cause malignancy. In this context, the chromosomal abnormalities induced by these viruses, and their ability to stimulate proliferation in normal cells, might be important determining factors in malignancy.

1.2.3 RNA TUMOR VIRUSES

In 1910, Rous (1910) first demonstrated that an avian neoplasm could be transmitted to other fowl by cell-free tumor extracts. However, over the next 5 years he was able to isolate a sarcoma-inducing virus from only 3/60 different tumors following serial transplantation in syngeneic chickens (see review by

Gardner, 1980). This finding was independently confirmed when a sarcoma virus was isolated from a transplanted chicken myxosarcoma (Fujinami and Inamoto, 1914). These viruses later became collectively known as avian sarcoma viruses (ASV). However, it was extremely puzzling that tumor viruses could be isolated from only a small proportion of tumors, and in fact only those sarcomas which were serially passaged gave rise to infectious transforming virus. It was not for another 60 years that this observation was satisfactorily explained.

Today, the general weight of evidence suggests that RNA tumor viruses with transforming capability are derived from recombinants between nontransforming leukemia (helper) viral RNA and cellular transforming genes (see reviews by Fischinger (1980) and Stephenson (1980)). This was indicated both by the results of molecular hybridisation studies and by the observations that transforming viruses frequently encoded some but not all leukemia virus structural components. These viral or cellular genetic sequences associated with transformation are in general designated 'onc' or 'src' genes. A model such as this explained why isolation of virus particles from sarcomas had been difficult, as isolation was dependent on both the presence of the helper virus and the recombination of virus genes with the src gene from the host chromosome.

For transformation or tumorogenesis to occur then, this model predicted that continuous src gene expression would be required. However, it was only quite recently that evidence was obtained

for a src gene product (protein). The first convincing demonstration of an ASV src gene coded protein resulted from studies by Brugge and Erikson (1977), in which sera from rabbits bearing transplantable ASV induced tumors were shown to immunoprecipitate a 60,000 dalton (60K) protein ($pp60^{src}$) from Schmidt-Ruppin ASV transformed chicken and hamster cells. Expression of this protein was temperature dependent in chicken cells infected with a temperature-sensitive (ts) transformation mutant (ts NY68), and was absent in uninfected cells.

The viral coded nature of ASV $pp60^{src}$ was confirmed by the synthesis of an analogous 60K protein in a cell free reticulocyte lysate using either 35S ASV genomic RNA or 20-24S poly (A) - containing ASV RNA as message (Purchio et al., 1977; Beemon and Hunter, 1978). Interestingly, this $pp60^{src}$ protein possessed protein kinase activity as immunoprecipitates containing this protein were shown to catalyze the phosphorylation of immunoglobulin heavy chains (Collett and Erikson, 1978; Levinson et al., 1978). Immunoprecipitates of extracts of normal cells were nonreactive in this assay. In addition, this $pp60^{src}$ protein kinase was found to be unusual as it specifically phosphorylated tyrosine residues (Hunter and Sefton, 1980) whereas most protein kinases phosphorylate serine or threonine residues (see Weller, 1979).

This demonstration of protein kinase activity associated with a viral transforming gene which was similar to a cellular gene (see Hunter, 1980) provided an attractive explanation of how a single gene product might cause marked changes to normal cells

(see Chapter 5 for brief discussion of the amplification effects of phosphorylation; see also Weller, 1979) which would result in the induction of cancers. One difference between normal and transformed cells was that the viral $pp60^{src}$ was present at about 50 times the level of its cellular counterpart (see Hunter, 1980). Thus cancer could be caused simply through a dosage effect. Furthermore, transformation by RSV resulted in a marked increase in the level of phosphotyrosine in protein reflecting the high levels of the virally coded protein kinase (Sefton et al., 1980), which appeared to result predominantly from a phosphorylation of a 36K polypeptide (Radke et al., 1980; Erikson and Erikson, 1980). This protein in its phosphorylated state might affect a number of cellular processes which would give rise to cancer.

Aside from the original isolates of avian sarcoma viruses from chicken sarcomas (Rous, 1910; Fujinami and Inamoto, 1914) many other RNA tumor viruses have been isolated from chickens and also from mice, cats and several types of primates (see review by Gardner, 1980). All such virus isolates come from animal tumors or leukemias, and many have been shown to transmit the oncogenic phenotype (see review by Gardner, 1980). Thus the evidence that RNA tumor viruses cause cancers in animals is very strong.

Perhaps the best evidence to date which provides evidence for RNA tumor viruses playing a role in human cancers comes from some very recent studies in Japan. Sero-epidemiological investigations in Southwestern Japan showed that specific antibodies

directed against type C RNA virus particles (from cultured T cell leukemia cells) reacted with cells from all patients (44) with adult T cell leukemia and with 32/40 patients with malignant T cell lymphomas (Hinuma et al., 1981). This area is endemic for adult T cell leukemia. 26% of healthy adults examined from the endemic area also reacted with this antibody but very few people from non-endemic areas reacted with the antibody. These data and two reports of isolations of new type C RNA tumor viruses from patients with T cell leukemia (Poiesz et al., 1980; Poiesz et al., 1981) and specific antibodies against viral glycoproteins, not evident in healthy controls (Kalyanaraman et al., 1981), support a role for human RNA tumor viruses in human cancers.

Other studies also support this view. Larsen et al.,(1975) for example, were able to find poly(A) - containing RNA capable of hybridising to a complementary DNA (cDNA) probe prepared from Moloney murine sarcoma virus in peripheral blood lymphocytes from 22/46 tested leukemic patients. Similarly, sequences complementary to a cDNA probe prepared from the Rauscher murine leukemia virus were found in the tissues of 2/8 leukemic patients (Aulakh and Gallo, 1977). Also,sustained release of virus particles with the properties of a mammalian retrovirus from cultured leukemic cells of a patient with acute myelogenous leukemia has been reported (Gallagher and Gallo, 1975). These reports all suggest an association between RNA tumor viruses and some human cancers. In addition, several other reports contain similar evidence to that (see review by Kaplan (1980)).

Summary

The data implicating RNA tumor viruses as etiological agents in cancers is very good from animal studies and appears to be steadily mounting for some human cancers. The mechanism by which these viruses would appear to exert their transforming effect is through the continuous expression of a transforming (src) gene derived from the cell genome, the product of which is a phosphotyrosine protein kinase. This would act to presumably modify a number of cellular functions, the net result of which would be to cause normal cells to become neoplastic.

1.2.4 PAPOVAVIRUSES

The papovaviruses, which include two of the most well-studied of the DNA tumor viruses, polyoma and SV 40, have been used as a major model system for the study of virus induced cellular transformation (for reviews see papers in Tooze (1980) and Ziff (1980)). SV 40 and polyoma also transform a variety of cell types and induce tumors in experimental animals, usually hamsters (SV 40) and mice (see reviews by Sambrook, 1972; Rapp and Westmoreland, 1976; and review papers in Tooze, 1980).

In 1971 two human papovaviruses were isolated (Gardner et al., 1971; Padgett et al., 1971) which led to much speculation that papovaviruses might be responsible for causing some human cancers. In this section of the thesis, I intend to concentrate on studies with the two human papovaviruses (BK virus (BKV) and JC virus (JCV)) which have investigated a role for these viruses in human

cancers and cancers in experimental animals. In Chapter 4 the evidence concerning the role of SV 40 in in vitro transformation and tumorogenicity is reviewed, and this evidence is discussed in terms of the specific viral gene products required to induce and maintain a transformed phenotype. For additional information refer to Tooze (1980).

The oncogenicity of both BKV and JCV has been demonstrated clearly in laboratory animals, particularly in Syrian hamsters. For example, Corallini et al. (1979) found that a large variety of tumors formed after intravenous inoculation of BKV and JCV into weanling hamsters. The same group reported that BKV also produced tumors in newborn mice after intracerebral inoculation (Corallini et al., 1977).

JCV is a highly neurooncogenic virus which after intracerebral inoculation appears to induce a number of different malignant gliomas, including medulloblastomas, glioblastomas, undifferentiated primitive gliomas, papillary ependymomas and pineocytomas, as well as an occasional meningioma (see Walker et al., 1973; Padgett et al., 1977). One particularly interesting observation was that JCV could induce malignant gliomas in adult nonimmunosuppressed owl monkeys (London et al., 1978). To date this is the only report that a human papovavirus is oncogenic in any kind of primate.

The observations mentioned above suggest that the human papovaviruses could play a role in tumor induction. However,

Corallini et al., (1976) tested the sera from 952 patients with various kinds of tumors and unlike the animal studies no correlation was found between prior infection with BKV and the occurrence of cancer. Indeed only a proportion of patients tested showed antibodies to BKV. Two or three other serological studies gave similar findings (see review by Padgett, 1980). The same group also screened these 952 cancer patients for BKV anti-T antibodies (the T antigens are thought to play an important role in the induction of tumors in experimental animals; see discussion of the function of the T antigens in SV 40 in Chapter 4) and found only 11 patients were positive, however, 4/501 healthy controls were also positive. In 4 other studies of cancer patients (see review by Padgett, 1980) no positive anti-T antibodies were found in a total of 268 patients tested. These results suggest that human papovaviruses do not induce tumors in man, unless they do so by a mechanism in which there is little or no accompanying production of T antigens. Searches for integrated papovavirus genome(s) in DNA extracted from human tumors and tumor cells have also produced very few positive results. For example, out of 197 human tumors or cell lines tested either by DNA/RNA hybridisation or DNA/DNA reassociation kinetics, no papovavirus (BKV) DNA sequences were detected (see review by Padgett, 1980).

Summary

Although there is no strong association between human tumors and papovaviruses, evidence from animal studies suggests that these viruses could contribute to some human cancers. Thus, either

papovaviruses do not cause human cancers, or they do occasionally cause cancers in some way which does not always require continued synthesis of viral specific proteins or the continued presence of viral nucleic acid sequences. There is at least some evidence that this is possible, as SV 40 does cause cellular chromosome alterations (see references cited in Sections 4.1 and 4.1.1), and both SV 40 and polyoma virus sometimes induce tumors in hamsters which do not synthesise detectable amounts of the gene products (T antigens) thought to be essential for tumorogenicity (Black and Rowe, 1965; Israel et al., 1980; Moore et al., 1980). The particular viral contributions to tumorogenicity might depend on the various selection conditions for transformation or tumor cell growth. Some of the results presented in Chapter 4 are discussed in terms of this framework. Thus papovaviruses could occasionally be a risk factor in the development of some cancers.

1.2.5 ADENOVIRUSES

Another group of DNA tumor viruses which has been investigated in depth is the adenoviruses. The 31 adenovirus serotypes isolated from man have been divided into 3 major groups (A, B and C) depending on their relative abilities to form tumors by direct inoculation into newborn hamsters. However, serotypes from both the oncogenic (A and B) and non-oncogenic (C) groups have been shown to transform cells of different types. For example, adenovirus type 12 (Ad 12), an oncogenic serotype, and adenovirus type 2 (Ad 2), a nononcogenic serotype, both transform rat cells in tissue culture with equal frequency (Ginsberg et al., 1974;

Gallimore, 1974). Cells transformed by oncogenic adenoviruses form tumors in syngeneic animals, whereas only some cells transformed by nononcogenic adenoviruses form tumors when inoculated, and it is usually necessary to immunosuppress, even in a syngeneic host animal (Gallimore, 1972).

In a comprehensive review of the characteristics of adenovirus induced transformed and tumor cells, McDougall et al.,(1977) concluded that there were two stable characteristics common to all these cells. These were the continued expression of the adenovirus-coded T antigens (coded from the transforming region of the virus, early region 1; see Sections 1.4, 3.1, 3.4, 4.1.2, and 4.4) and the retention of at least part of the virus genome (early regions 1A and 1B; see Section 1.5 and Chapter 4), covalently linked (integrated) to host DNA. McDougall's conclusions once again provide strong support for a requirement for continued expression of some virus (transforming) gene to induce and maintain the transformed or tumorogenic phenotype, as is demonstrably the case for RNA tumor viruses (see Section 1.2.3). Other data however suggest that the situation is more complicated than McDougall et al.,(1977),would seem to imply, and as for herpes-viruses there seem to be a number of factors affecting whether or not infection by adenoviruses will result in tumor development.

Many tumors induced in hamsters by chicken embryo lethal orphan (CELO) virus (an avian adenovirus), and transformed cells selected by growth in soft agar, were negative for the synthesis of CELO tumor (T) antigens (Asch et al., 1979). Both T protein

positive and T protein negative cell lines derived from these tumors, were examined for the presence of integrated viral DNA sequences (May et al., 1978). Both types of cells were found to contain integrated viral DNA sequences, although there was a deletion detected in the non T protein producing cells which was tentatively interpreted to include the T antigen gene. Results of these experiments suggest that the generalities of McDougall et al., (1977) might not always be correct.

Bellett and Younghusband (1979) isolated a number of mouse cell variants in agarose after infection with adenovirus type 5 (Ad 5). 14/20 Ad 5 induced transformed clones did not synthesise T antigens and 2 of these clones also did not contain detectable amounts of covalently linked viral DNA. Of the other 6 clones some cells in each were T antigen positive when first tested but were negative when retested 8 weeks later. All cloned variants were tumorogenic in athymic and in normal syngeneic mice. These results suggest, in contrast to the requirements suggested by McDougall et al., (1977), that neither integrated viral DNA nor continued expression of the Ad 5 T antigens are mandatory requirements for transformation or tumorogenicity. A similar lack of correlation between synthesis of T antigens and tumorogenicity has been reported by Paraskeva and Gallimore (1980) for Ad 12 induced transformants from rat liver epithelial cells. They isolated 8 Ad 12-transformed cell lines, 3 of which were T antigen negative, and all of which formed tumors in newborn syngeneic rats. The T antigen positive transformants all produced anaplastic epithelial tumors, whereas the T antigen

negative lines all produced adenocarcinomas. Thus the specific viral requirements for transformation and tumorogenicity probably depend on a number of factors which affect the growth conditions of the cells which eventually give rise to tumors. Whilst specific adenovirus gene products might be required to alter cell growth properties in ways which are relevant to tumor formation, continued presence of virus DNA, or virus gene expression, might not always be required. The effect of selection conditions in vitro on adenovirus and SV 40 gene expression and its relationship with tumorogenicity is discussed in some detail in Chapter 4 as well as the viral genetics of transformation. Other evidence that there might be several factors affecting the induction of tumorogenicity by adenovirus, comes from experiments with tumor promoting agents in which it was shown that these agents increase transformation frequency after adenovirus infection (Fisher et al., 1978), and also enhance the ability of such transformants to exhibit anchorage independent growth (Fisher et al., 1979).

In spite of the fact that the viral, cellular, and environmental contributions to adenovirus induced tumorogenicity are not particularly well understood, the evidence that the virus or virus transformed cells are oncogenic when inoculated into rodents is well-acknowledged (Trentin et al., 1962; Gallimore, 1972; Gallimore, 1974; Ginsberg et al., 1974; Bellett and Younghusband, 1979; Paraskeva and Gallimore, 1980; other references cited in Chapters 3 and 4). Because an association between adenoviruses and transformed or tumor cells cannot

always be demonstrated, experiments designed to establish whether adenoviruses play an etiological role in human cancers are difficult to interpret using the usual criteria (serology, nucleic acid hybridisation; see previous sections). The evidence to date suggests that adenoviruses do not cause human cancers, or if they do it is a rare event. The studies from Green's laboratory are important in this context (Green and Mackey, 1977). These workers used cDNA probes from the transforming genes in early region 1 (see Section 1.5) of adenovirus groups A and C which were hybridised to more than 100 human gastrointestinal or lung tumors. In no case was any significant hybridisation between the cDNA probes and these human tumors observed. Maitland et al., (1981) however did find approximately 2 to 3 fold higher association of an Ad 2 DNA (genome) probe with RNA from human cervical tumors, than with normal cervical biopsies. This technique is more sensitive than that used by Green and Mackey (1977) but there was a high background hybridisation (7% with phage lambda), and normal cells also showed some nucleic acid sequence homology with Ad 2 DNA. It is therefore difficult to interpret this experiment, but even at best it only demonstrates a small association between human adenoviruses and cancers. An association between Ad 2 DNA and some normal human tissues has previously been reported by this group (Jones et al., 1979), as well as cellular proteins related to Ad 2 antigens.

The data from these kinds of experiments suggest that adenoviruses are not important in the generality of human cancers. However,

since some experimental adenovirus induced tumors and transformed cells lack demonstrable viral T antigen and integrated transforming genes, adenoviruses cannot be excluded as risk factors in some cancers.

1.2.6 SUMMARY AND CONCLUSIONS

The studies reviewed in previous sections of this chapter have provided both direct and indirect evidence that some viruses play a role in causing cancers in animals and man. This evidence comes from studies with experimental animals and from serological and epidemiological investigations in man, as well as numerous studies in vitro. The most convincing evidence for a viral etiology of human cancers is for BL and NPC, where EBV is strongly implicated. However, even in these cases other factors are clearly involved. Such studies increasingly suggest that there are many factors contributing to the induction of tumors, and that tumor viruses might act as "risk factors" in the development of some human cancers without being their sole cause.

The molecular basis of tumorogenesis is unknown, but there is evidence that a virally coded protein is sometimes continuously required to modify cellular growth controls (e.g. RNA tumor viruses), while in other cases it may be transiently required to modify cells in a permanent way, followed by selection of the tumorogenic phenotype (e.g. CELO virus).

One means of investigating how viruses might be involved in cancer development, is to study the effects candidate viruses

have on cells at a molecular level. It might then be possible to correlate such changes mediated by viruses (if any) with transformation and tumorogenicity. The experiments reported in this thesis represent such a study in which the effects of adenovirus infection on normal cell growth controls is studied. In the next section some aspects of the mammalian cell cycle and eukaryote DNA replication are discussed.

1.3 CONTROL OF CELLULAR PROLIFERATION

1.3.1 INTRODUCTION

The mammalian cell growth cycle has been divided into 4 distinct phases consisting of DNA synthesis (S phase) and mitotic phases (M), which are separated by 2 gaps, G_1 and G_2. G_1 separates M from S and G_2 separates S from M. Certain crucial events are thought to take place during G_1 which regulate entry into S phase (G_1 to S phase transition), and similarly G_2 acts as a regulatory phase for entry into mitosis. If these crucial events do not take place then no further cell cycle progression occurs and cells become "quiescent", i.e. they cease cycling and become arrested. This arrest occurs at specific points in the cell cycle and is discussed in the following section.

The ability of cells to shift into a noncycling but viable state is an important characteristic of normal cells grown in tissue culture, and might be an important factor in allowing cells to differentiate in vivo, as nearly all cells in an animal have G_1

(diploid) DNA contents. This ability to arrest may also serve as an important distinguishing feature of normal as opposed to transformed or tumor cells, which are often undergoing rapid growth, particularly during the invasive stage of a metastasising tumor.

The experiments described in this thesis are primarily concerned with the effect of adenovirus infection on the regulatory events in G_1 which lead to the initiation of cellular DNA replication (see Chapters 3, 5 and 6). However, some parts of the thesis also examine the effect of adenovirus infection on other phases of the cell cycle (see Chapters 4 and 6), as well as the effect of a drug (cycloheximide) on the rate of cell cycle progression (see Chapter 6). Thus, in this section of the thesis I have discussed some (but not all) important characteristics of the G_1 phase; the mechanism of eukaryote DNA replication; and briefly the regulatory events which occur during G_2 + M.

1.3.2 G_1 PHASE

Unlike the other phases of the cell cycle which have a relatively constant duration, the G_1 phase appears to be highly variable in its time span, and the variation that occurs in cell generation time can be accounted for solely by variation in the length of the G_1 phase (see Prescott, 1976). For this reason, and also because when cell proliferation slows down during unfavourable growth conditions, there is a selective accumulation of cells in the G_1 phase of the cell cycle, G_1 has been considered as the major phase of the cell cycle that regulates the rate of cellular

proliferation. This section considers only this aspect of the G_1 phase and does not attempt to review the many G_1 related biochemical events that occur. These are reviewed adequately in Prescott (1976), Gurley et al., (1977), and Pardee et al., (1978).

If G_1 is the critical regulatory period for cellular proliferation then some signal must occur during this phase which triggers a sequence of events which leads to the onset of DNA synthesis and subsequently the remainder of the cell cycle. If this signal does not occur no cell cycle progression would take place and cells would become G_1-arrested at what Pardee (1974) calls the "restriction point" (this term is used throughout this thesis).

The original experiments by Pardee (1974) provided the basis for this concept of "restriction point" when he showed that several "physiological" regulators of cell cycle progression blocked cell proliferation at approximately the same point in the cell cycle (in G_1). Thus serum starvation, amino acid deprivation, paperavine and dibutyryl cyclic AMP (dbc AMP) treatment all blocked cells at the restriction point. On the other hand, RSV transformed cells appeared to be insensitive to restriction point control.

More recently, other studies have suggested that there is more than one point in G_1 at which cellular proliferation is regulated. Pledger et al., (1977) studied the induction of DNA synthesis in mouse cells treated with platelet derived growth factor (PDGF; released during clotting) and platelet poor plasma (PPP)

found in defibrinogenated blood. PDGF was shown to induce cells to become competent to enter S phase, but alone was not sufficient to effect entry into S phase. In order for this to occur, continuous exposure to PPP was required. Subsequent studies showed that PDGF deficient cells became blocked 6 h before the onset of DNA synthesis (after the low serum arrest point), and provided evidence for another restriction at the G_1/S phase boundary (Pledger et al., 1978). A similar result had been previously obtained by Temin (1971) who showed that chicken cells were committed to enter DNA synthesis 4 h prior to the initiation event. Thus unlike the single restriction point suggested by Pardee (1974) there would appear to be several such points, which define a sequence of events leading to DNA synthesis in mammalian cells.

Several studies reviewed in Gelfant (1977; 1981), and results presented in this thesis (see Chapter 5) have provided considerable evidence for multiple arrest (restriction) points. Such data are consistent with a linear sequence of events being required for cellular proliferation to occur; it would seem that several signals might be required for complete transition through the G_1 phase into DNA synthesis.

Viral transformation of cells appears to modify the way cells respond to these regulatory controls, as was indicated by the lack of restriction in RSV transformed cells reported by Pardee (1974). Studies by Scher et al., (1978) and Stiles et al., (1979) have provided additional evidence for a relaxation of

restriction point controls in virally transformed cells. Normal human or mouse fibroblasts proliferated poorly in medium deprived of PDGF (Scher et al., 1978) or PPP (only mouse cells tested, Stiles et al., 1979) but grew well upon readdition of these growth factors. By contrast, when these same cells were transformed with SV 40 or several different RNA tumor viruses, they became insensitive to PDGF deprivation (Scher et al., 1978), and for SV 40 transformed mouse cells, also insensitive to PPP deprivation (Stiles et al., 1979). PPP is now thought to be composed of somatomedins, based on studies with normal and hypophysectomized animals (Stiles et al., 1979). Thus these transforming viruses probably contain genetic information which can directly reduce the level of both competence and progression factors required for G_1 to S phase progression, which might be relevant to viral tumorogenicity.

One mandatory requirement for the G_1 to S phase transition to occur is protein synthesis. This was shown by Pledger et al., (1978) and also by several other groups (see Prescott, 1976). Studies from Pardee's laboratory have suggested an important regulatory role for protein synthesis in cell cycle proliferation.

Rossow et al.(1979) treated Swiss 3T3 and balb/c 3T3 cells with cycloheximide (a potent inhibitor of protein synthesis), at concentrations which cause only partial inhibition of protein synthesis, and then examined cell growth rate, cell doubling time, and position in the cell cycle by flow cytometry. Cycloheximide produced a lowered growth rate and therefore an

increased doubling time, and an increase in the proportion of cells in the G_1 phase of the cell cycle. This latter result was obtained with exponentially growing cells and with cells which had been serum arrested prior to the addition of cycloheximide. On the basis of these experiments, and mathematical analysis of transit time as a function of protein synthesis, Rossow and colleagues concluded that in the G_1 phase there is a highly labile serum dependent protein which regulates the passage of cells through the G_1 phase to DNA synthesis. If this protein is not in sufficient intracellular concentration cells become arrested in G_1.

Detailed experiments by Schneiderman et al., (1971) using short pulses of a high concentration of cycloheximide, also provided good evidence for a labile initiator protein. When cells were blocked in late G_1 by cycloheximide treatment, the delay in entry into S phase was longer than the duration of the cycloheximide pulse. Such results suggested that certain proteins synthesised prior to cycloheximide treatment had decayed and had to be resynthesised before DNA synthesis could be initiated. Some of these experiments and others are discussed in Section 6.1.

In virally transformed or tumorogenic cells the requirements for protein synthesis appeared to be modified. Medrano and Pardee (1980) showed that a number of SV 40 transformed and tumorogenic cells were much less sensitive to inhibition of protein synthesis than normal cells, as determined by flow cytometric analysis of DNA content. Such results suggested that there was a reduced

requirement for one or more of the cellular initiator proteins in transformed cells. Presumably, some virally coded gene product substitutes for the cellular proteins in order to maintain cell cycle progression in transformed cells. More evidence for these different protein synthesis requirements in transformed cells, and its relationship with tumorogenicity, is discussed in Section 6.1. The regulation of cell cycle progression by protein synthesis is investigated in Chapter 6 in normal and adenovirus infected rodent cells, and as for transformation, infection appears to modify the protein synthesis requirements for cell cycle progression.

In addition to the studies discussed above, more direct evidence for S phase initiator proteins has been obtained from cell fusion studies. Rao et al., (1977) fused late G_1 cells with other cells taken from early, middle and late G_1, and then at different times measured DNA synthesis. Their data demonstrated that a more rapid onset of DNA synthesis occurred when two late G_1 cells were fused together than from fusion of cells from earlier parts of G_1. This result suggested that there was a progressive accumulation of an inducer of DNA synthesis through the G_1 phase of the cell cycle.

Yanishevsky and Prescott (1978) using Chinese hamster ovary cells showed that fusing late S phase cells with G_1 cells induced DNA synthesis in G_1 nuclei, and that the autoradiographic patterns produced were characteristic of "early" S phase nuclei. This experiment suggested that there is probably a single inducer factor (not one for early DNA replication and one for late

replication) and that it is present throughout the duration of S phase. The stability of the inducer factors implied in these two studies would seem however to be inconsistent with the lability of inducer factors suggested from other studies. However, it is possible that there may be labile G_1 initiators, but stable S phase inducers of DNA synthesis which would allow DNA synthesis to proceed once it had been initiated.

In summary, there appears to be an ordered sequence of events required for complete traverse of the G_1 phase into DNA synthesis, as well as synthesis of certain S phase initiator and inducer proteins. In virally transformed cells some of these regulatory events and initiator proteins appear not to be required. Thus transforming viruses directly alter some of the controls of cellular proliferation.

The next section discusses some of the events which take place during DNA synthesis, with particular reference to mammalian cells.

1.3.3 REPLICATION OF THE MAMMALIAN CELL CHROMOSOME

1.3.3.1 STRUCTURE AND REPLICATION OF DNA

With the discovery of the double helical structure of DNA (Watson and Crick, 1953) came the realisation of how the DNA might replicate in a semi-conservative manner, later described by Meselson and Stahl (1958). Recent advances have resulted in considerable knowledge of the enzymology and mechanisms of

DNA replication and have been reviewed in the past few years (Denhardt, 1977; Alberts and Sternglanz, 1977; Hand, 1978; Kornberg, 1980; DePamphilis and Wassarman, 1980). The same basic principles of DNA replication apply to both prokaryotic and eukaryotic cells and to their viruses; however, the detailed mechanisms in each individual cell system can be as variable as the biology of the system itself. In this section I will discuss some general features of DNA structure and replication, and in the following section I will give a more detailed account of some of the events which take place at the replication fork of mammalian cells. This discussion is pertinent to the studies on adenovirus induced cellular DNA replication reported in this thesis (see Chapter 3 in particular).

With improvements in the techniques of isolation of intact DNA from cells, double-stranded DNA has been found to be organised into specific toroidal arrangements leading to helical secondary and tertiary structures (Worcel and Burgi, 1972; Oudet et al., 1975; Worcel and Benyajati, 1977). Eukaryotic DNA, including that of animal viruses, is structured into DNA-protein complexes in the chromatin. The eukaryotic cell chromosome consists of "nucleosome" subunits made up of an octamer of histone proteins protecting about 140 to 240 base pairs of DNA with a nucleosome core of 140 base pairs. Between each nucleosome unit is a spacer region that is variable in length between cell types, and even within a single cell type. The nucleosome structures and chromatin have been recently reviewed by McGhee and Felsenfeld (1980).

Replication of double-stranded DNA is semi-conservative, with the progeny molecule containing one strand of parental DNA and one strand of newly replicated DNA. The parental DNA provides a template for DNA replication by DNA polymerases which synthesise DNA by addition of 5'-mononucleotides in the 5' to 3' direction. Three major polymerases have been isolated from *E. coli* (Kornberg, 1980) and from mammalian cells (Weissbach, 1975; Denhardt, 1977) which in the latter case have been designated α, β and γ. Other species of DNA polymerases found in vertebrate cells include viruses induced polymerases (Weissbach, 1975); terminal deoxynucleotidyl transferase, which is a template independent polymerase (Baltimore, 1974; Kung et al., 1975); and the unique enzyme class, the RNA-dependent DNA polymerases of the RNA tumor viruses (Temin and Baltimore, 1972). It would appear from many experiments in recent years that DNA polymerase α is the major replicative enzyme in mammalian cells (Castellot et al., 1979; Hubscher et al., 1978; Krokan et al., 1979; Wist, 1979), whereas the β polymerase is thought to be involved in repair replication (Castellot et al., 1979; Hubscher et al., 1978), and the γ polymerase replicates mitochondrial DNA (Bolden et al., 1977). The α polymerase in association with the γ polymerase is thought to be responsible for synthesising adenovirus DNA (Krokan et al., 1979; Habara et al., 1980).

Due to the antiparallel polarity of the strands in double-stranded DNA, it is clear that within a replication fork in which both parental strands act as templates, one strand must replicate in the 5' to 3' direction overall, while the other replicates 3' to 5'. To explain replication of a strand in the overall 3' to 5' direction

Revised paragraph, p. 37

The short initiator RNA is synthesised by primer-independent polymerases which are in some cases sensitive to rifampicin and other inhibitors that inactivate RNA polymerases required for transcription. The best characterised example of these enzymes is the rifampicin resistant dna G gene of *E. coli*, which has been named primase. However, similar enzymes have been implicated in the synthesis of initiator RNA in some animal viruses. This area is reviewed by DePamphilis and Wassarman (1980). The next section is concerned with some of the details of events occurring at the replicating fork.

by polymerases that can synthesize only in the 5' to 3' direction, Okazaki and co-workers (Okazaki et al., 1968; Sugino and Okazaki, 1972) proposed the synthesis of short strands in the 5' to 3' direction (Okazaki fragments) which are subsequently ligated to form the mature strand. As the known DNA polymerases require a free 3'-hydroxyl group as a primer for elongation, it was proposed that short oligoribonucleotides are synthesized as a primer for the Okazaki DNA fragment, then removed and the gap filled by repair synthesis using the 3'-hydroxyl from the previous fragment as a primer. In this way, replication of the strand proceeds overall in the 3' to 5' direction. Direct experimental evidence for this mechanism has been obtained (for example, Machida et al., 1977; Okazaki et al., 1975; Sternglanz et al., 1976). Initiator RNA has been reported for *E. coli* DNA replication (Okazaki et al., 1975), papovavirus DNA replication (Magnusson et al., 1973; Reichard et al., 1974), and for mammalian cell DNA replication (Waqar and Huberman 1975; see review by DePamphilis and Wassarman, 1980).

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1.3.3.2 A CLOSE-UP OF THE EUKARYOTE REPLICATION FORK

In order for initiation of eukaryotic DNA replication to occur, helix destabilising proteins and DNA-unwinding enzymes must first bind to the origin of replication (initiation site). Helix destabilising proteins prevent reannealing of complementary DNA strands by binding cooperatively to single-stranded DNA, and DNA-unwinding enzymes that are single-stranded DNA-dependent ATPases that catalyze unwinding of duplex DNA (Alberts and Sternglanz, 1977). Both classes of proteins have been isolated from different organisms including rodent and human cells and vaccinia virus (see review by De Pamphilis and Wassarman (1980).

Denaturation of the replication origin by binding of destabilising and unwinding proteins allows the subsequent binding of DNA polymerase α, RNA polymerase for priming, and DNA ligase I for gap filling. Once these enzymes have bound to the DNA, DNA synthesis proceeds bidirectionally from the origin in a semidiscontinuous manner (Huberman and Riggs, 1968). That is, continuous synthesis on the leading strand (5' to 3' direction) and discontinuous synthesis (Okazaki synthesis) on the lagging strand (in overall 3' to 5' direction). Other proteins are also involved in eukaryote DNA replication (see DePamphilis and Wassarman, 1980) but the ones discussed above are those for which there is most evidence.

Unlike *E. coli*, where DNA replication is initiated at a single origin (Cairns, 1963), eukaryotic DNA synthesis is initiated at multiple origins (Huberman and Riggs, 1968), thus forming several

replicons. Some, but not all replicons are initiated synchronously, which give rise to clusters of replicating units (see references in Hand, 1978). Some replicons are extremely large as demonstrated by DNA fiber autoradiography. For example, Yurov and Liapunova (1977) found a range of 40 to 280 μm with an average value of 120 μm in chinese hamster cells. The number of replication origins and their times of activation vary, depending upon the cell type, as does the rate of replication at the fork during S phase, and with the addition of extrinsic factors such as estrogen (see Denhardt, 1977).

The nature of the origin of DNA replication itself has been examined in a number of chromosomes using DNA sequencing techniques. For eukaryotes however, the evidence derives solely from studies on the origins of replication of the animal viruses SV 40, polyoma virus and adenovirus (Subramanian et al., 1977; Friedman et al., 1978; Steenburgh et al., 1977). Most such origins have sequences such that considerable secondary structure can be proposed. For initiation of Okazaki DNA fragments, the priming step by RNA polymerase does not appear to occur at any specific sequence, but some evidence suggests that the eukaryotic nucleosome is the structure which provides the recognition signal for Okazaki DNA fragment synthesis. For example, eukaryote Okazaki fragments are about the same length (200 base pairs) as the nucleosome structure. This area is reviewed by Hand (1978).

In SV 40 transformed chinese hamster cells, Martin and Oppenheim (1977) showed that the initiation intervals were smaller than in untransformed cells, suggesting that there is another class of

initiator sites which are not usually utilized during normal DNA replication. This might be an important observation concerning the regulation of cellular proliferation in virally transformed cells.

The mechanism for completion of replicons is currently unknown, however this event does appear to play a role in the regulation of DNA replication. The rate of merging of two or more replicons is slower than the rate of replication fork movement which suggests that completion of replicons is a rate-limiting step of chromosome replication (see references in DePamphilis and Wassarman, 1980). Possibly a replicon is only completed when all replicons in that particular cluster are ready to merge.

1.3.4 G_2, MITOSIS, AND DIVISION

The G_2 phase of the cell cycle is primarily the phase during which preparations for mitosis occur and protein synthesis is required in order to advance to mitosis (see Prescott, 1976). However, there is evidence for a weak control point of cell cycle progression in G_2, as various conditions have been reported to arrest cells there; for example dibutyryl cyclic AMP in V79 cells (Stambrook and Velez, 1976) and many drugs used in cancer chemotherapy (see review by Pardee et al., 1978).

Tubulin, a component of the mitotic spindle, is synthesised in high amounts during G_2, and phosphorylation of this protein has been observed to occur to a greater extent than any other phase of the cell cycle (Piras and Piras, 1975). Histone protein

phosphorylation has been observed to increase during G_2 as well (Gurley et al., 1977; Pardee et al., 1978). The event that initiates mitosis is unknown, but it is thought to be dependent on synthesis of one or more cytoplasmic inducing factors (probably proteins; see Prescott, 1976). For example, fusion of mitotic cells with interphase nuclei provokes premature condensation of chromosomes in the interphase chromatin (Rao and Smith, 1976). Cell division (cytokinesis) rapidly follows mitosis and cells return to the G_1 phase of the cell cycle.

1.4 ADENOVIRUS MOLECULAR BIOLOGY

1.4.1 INTRODUCTION

Although this thesis is not concerned with the molecular biology of adenoviruses per se, one of the reasons for choosing a group C adenovirus (e.g. Ad 5) for the experimental studies was that the molecular genetics of these viruses, particularly with respect to transformation, has in recent years been intensively investigated. Because of this, and because of the availability of well-characterised mutants which affect transformation, using such a virus to investigate cell cycle alterations and their relationship to transformation seemed a useful approach.

This section reviews some aspects of the molecular biology of adenoviruses which are related to transformation, and therefore discusses only the "early regions" which appear to be entirely responsible for transformation (Gallimore et al., 1974; see Sections 3.1, 4.1.2, and 4.1.3). The "early regions" are those

DNA sequences transcribed prior to viral DNA replication, after which late transcriptional units are expressed. This terminology is used by Philipson and Lindberg (1974).

1.4.2 ADENOVIRUS EARLY REGIONS

The human adenoviruses, which include Ad 2, Ad 5, and Ad 12 (see Section 1.2.5), are icosahedral in structure and contain a single, linear double-stranded piece of DNA of about 35,000 nucleotide-pairs. Attached to the 5'-terminus of each DNA strand is a covalently bound protein (Robinson and Bellett, 1974; Robinson et al., 1979) with a molecular weight of 55,000 daltons (55K) (Rekosh et al, 1977) which provides the priming function for DNA replication (Stillman and Bellett, 1978).

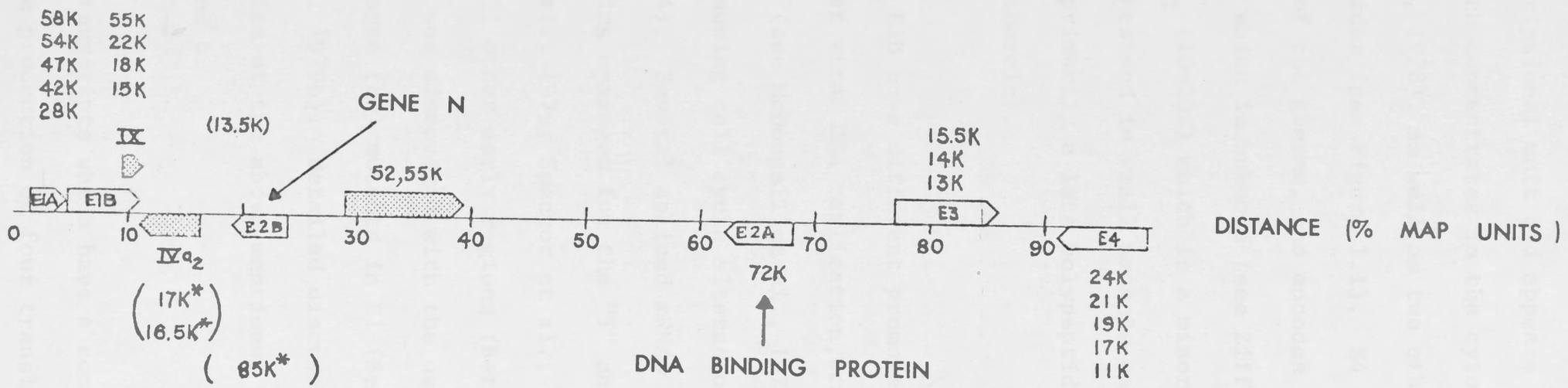
The regions of the adenovirus genome encoding early viral functions are located in 4 main blocks called E1, E2, E3 and E4 on the virus chromosome. This organisation is illustrated in Figure 1.1 for Ad 5. Early region 1 consists of two subregions, E1A and E1B, located at the left-hand end of the genome (0-4.5% and 4.5%-11.1% respectively, expressed in % map units; see Lewis et al., 1979) and is specifically responsible for cellular transformation (see discussion in Chapter 4 and references cited therein). Early region 2 consists of two subregions, E2A and E2B; E2A encodes the 72K DNA-binding protein which is located at position 62-68% on the Ad 5 genome (Stillman et al., (1981), and E2B codes for 3 polypeptides, one of which has a molecular weight of 87K and is the precursor of the 55K terminal protein (Stillman et al., 1981). E3 is located in the middle of

FIGURE 1.1

A simplified map of the genetic organisation of Ad 5. The regions of interest for the results described in this are early regions 1A, 1B, 2A and 2B (see text). The figures (0, 10, 20 ...) represent % map units and the other figures (accompanied by a 'K') refer to the molecular weights in thousands of polypeptides synthesised by mRNAs encoded by those regions. Those figures with a (*) beside them represent tentative molecular weight assignments. Transcriptional units pointed towards the right of the figure represent mRNAs coded by the 'r' strand; those pointed towards the left are coded by the 'l' strand.

This figure is modified from Stillman et al., (1981).

GENETIC MAP OF ADENOVIRUS TYPE 5



the late transcriptional unit and appears to encode a 14 K polypeptide which concentrates in the cytoplasm during infection (Persson et al., 1978), as well as two other small molecular weight polypeptides (see Figure 1.1). E4 is found at the far right-hand end of the genome, and encodes several polypeptides the function of which is unknown (see Ziff, 1980). In addition polypeptide IVa_2 (10-20%) which is a minor component of the virion, is synthesised in small amounts early after infection, although it is primarily a late polypeptide (Galos et al., 1979; and references therein).

Regions E1A and E1B have different promoters (see Ziff, 1980) and are essential for viral DNA replication, transformation and tumor formation (see McDougall et al., 1977), and also E1A is essential for causing cell cycle alterations (see results in Chapters 3 and 4). Several spliced mRNAs are encoded from these regions, including messages for the "T" antigens (Chow et al., 1979; Lewis et al., 1979; Spector et al., 1979). E1A regulates expression of all other early regions (Berk et al., 1979). This phenomenon was discovered with the use of specific deletion (d1) and host-range (hr) mutants in E1 (Berk et al., 1979; Jones and Shenk, 1979b). Detailed discussions of this region and the properties of the above-mentioned mutants, are found in Chapters 3 and 4.

E2A codes for transcripts which have a complex splicing pattern resulting in the production of four translatable mRNAs, one of which encodes the adenovirus DNA-binding protein (Chow et al.,

1979; Kruijer et al., 1981). This is an essential requirement for viral DNA replication (see Section 3.1). The splicing pattern of pre-mRNA processing becomes more complicated late in infection as the 5'-leader sequence is initiated at a different position on the Ad 2 genetic map, shifting from an initiation site at position 75 early to 72 and 86 late (see Ziff, 1980).

Recently, E2B has received considerable attention as experiments by Stillman and colleagues (1981) have implicated this region (11.2 to 31.5) as containing the gene coding for the adenovirus terminal protein. This region encodes a series of 3 mRNAs on the 1-strand extending from positions 30, 26 and 23 to 11.1. An 87K protein is synthesised from this region which is cleaved to the 55K terminal protein, possibly via a 62K intermediate. Peptide analysis shows all 3 proteins to be related. This region is interesting in that it overlaps the region coding for the gene N products at coordinates 19.8 to 23.5 to which the viral DNA-negative mutants ts 36 and ts 37 have been mapped (Galos et al., 1979). These mutants, which have altered transforming properties as well as being defective in viral DNA replication (see Section 3.1), are tested for cell cycle alterations in this thesis.

The regions of the adenovirus genome coding for the IVa_2 protein, and E3 and E4 also have complex splicing arrangements leading in the latter cases to the production of 6 and 5 mRNAs which differ considerably in initiation and termination sites, and in their internal sequences (Ziff, 1980). The functions of these regions are unknown and they are not essential for transformation.

Early transcription units appear to be sensitive to negative regulation as determined with the use of mutants in E1A (Berk et al., 1979; Jones and Shenk, 1979b; discussions in Chapters 3 and 4) and cycloheximide. Ad 2 infected cells treated with cycloheximide overproduce (or stabilise) early mRNA suggesting that protein synthesis is required to regulate transcription (Craig and Raskas, 1974). Aside from E1A, this negative regulation has been convincingly shown for the DNA-binding protein (in E2A). A ts mutant in this region (ts 125; see Section 3.1), which is defective in synthesis of the DNA-binding protein overproduces mRNA from E4, but has no effect on E1A and E1B, or on mRNA from E2A; thus it is not autoregulatory (Nevins and Winkler, 1980). Mutant ts 125 is also examined in this thesis, as it too has altered transforming properties (see Section 3.1).

1.4.3 SUMMARY

Adenovirus has a complex but versatile pattern of early gene expression, in part concerned with preparation for DNA replication. This has been investigated in detail with the use of mutants in the early regions, which have been found also to affect the ability of viruses to transform cells. These mutants, defective in E1A, E1B, E2A and E2B, have been used in this thesis to study the genetics of adenovirus-induced cell cycle alterations and their relationship to transformation.

1.5 EXPERIMENTAL APPROACHES USED IN THE THESIS

To study the molecular basis of oncogenesis beginning with transformed or tumorogenic cells is very difficult, as all the important molecular alterations have already taken place. However, the differences between normal and transformed cells do highlight areas which would seem to be of interest, to determine what molecular changes occur during the initiation events of transformation. One area which would seem to be of importance in this context, is the effect a transforming virus has on the controls of cellular proliferation early after infection (see Sections 1.2 and 1.3). These early events perhaps set the foundation for later development of malignant cells.

Experiments described in Chapters 3 and 4 investigate the effect of early region mutants on the induction of cellular DNA replication (Chapter 3), and on all phases of the cell cycle (Chapter 4). In the next two chapters, normal cellular proliferation is prevented (Chapter 5) or slowed (Chapter 6) with the use of different agents and the ability of Ad 5 to modify the effects of these agents is investigated. The results are discussed with a view to relating cell cycle alterations to transformation (Chapter 7).

CHAPTER 2

GENERAL METHODS

2.0 INTRODUCTION

In this chapter those methods which are routinely used throughout the thesis are described, including an outline of cell and virus growth procedures and a list of reagents used for specific procedures. Specific methods of DNA analysis by ultracentrfugation, microspectrophotometry, and flow cytometry and the cyclic nucleotide phosphodiesterase assay are described in the appropriate chapters later in the thesis (see Chapters 3, 4, and 5).

2.1 BIOLOGICAL METHODS

2.1.1 CELLS AND MEDIA

Primary cultures of C57Bl mouse embryo fibroblasts and PVG or Wistar rat embryo fibroblasts were prepared by the method described by Bellett and Younghusband (1979). Cultures of all cells were grown in "Autopow" medium (AP) (Flow laboratories) supplemented with 10% fetal calf serum (FCS) in 75 cm^2 plastic culture flasks (Falcon).

2.1.2 VIRUSES AND VIRUS GROWTH

Ad 5, ts 36, ts 37, and ts 125 were grown in monolayer cultures of human KB cells and titrated in human embryo kidney cells (HEK) by the fluorescent cell counting method (Philipson, 1961). Titres of ts mutants at the permissive temperature (32.5^{o}C) were generally about 10^9 infectious units (iu)/ml, which dropped

by 10^3 to 10^4 iu/ml when titrated at the nonpermissive temperature (39.5°C). dl 312, dl 313, and hr 7 were grown in 293 cells which are HEK cells transformed with a restriction fragment of Ad 5 containing early regions 1A and 1B (Graham et al., 1977). These mutants were titrated as described above on both HEK and 293 cells. Titres of these mutants were usually 10^8 to 10^9 iu/ml on 293 cells and once again 10^3 to 10^4 iu/ml lower when titrated on nonpermissive HEK cells. All virus inocula were stored at -70°C as crude cell lysates. Ad 5 and ts mutants were grown from stocks originally supplied by Dr J. Williams (Carnegie-Mellon University, Pittsburgh, Pennsylvania), and deletion mutants and 293 cells were originally supplied by Dr B. Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

2.1.3 GROWTH AND ARREST OF CELLS

In order to determine appropriate G_1-arrest conditions for stimulation experiments early passage mouse and rat embryo fibroblasts, BHK 21 cells, and HEK cells were seeded at an initial density of 4 to 7 x 10^5 cells into 50mm plastic petri dishes (Kayline). They were cultured for 1 day in AP + 10% FCS and then shifted to medium (AP) containing 0.2% bovine serum (BS). At indicated times cells were pulse-labeled with [^{3}H] thymidine to measure DNA synthesis (see Section 2.2.1), harvested, and measured for incorporation of radioactivity (Section 2.2.2). Results from this experiment (Fig. 2.1) showed that early passage mouse and rat fibroblasts were easily arrested as DNA synthesis

had declined to background levels in both cultures by 2 days after shifting to AP + 0.2% BS. They remained viable as they could subsequently be stimulated to synthesise DNA by re-addition of AP + 10% FCS 4 days (mouse) and 5 days (rat) after serum starvation.

By contrast, BHK 21 and HEK cells continued to show an increasing rate of DNA synthesis until they reached a plateau of DNA synthesis (Fig. 2.1). BHK 21 cells reached a plateau by 2 days after serum starvation, however HEK cells did not reach a constant level of DNA synthesis until about 1 week after serum starvation. At this time HEK cells had reached confluency and so appeared to be insensitive to the effects of AP + 0.2% BS. Thus for stimulation experiments mouse cells were arrested by culturing in low serum medium for 2 days; rat cells for 3 days; BHK 21 cells for 5 days; and HEK cells were grown to confluency in AP + 10% FCS for 7 to 8 days. After these time periods cells were then infected with Ad 5, mock infected, or serum stimulated (see Chapters 3, 5 and 6).

The next experiment was designed in order to verify that such culturing in low serum medium did indeed cause a selective increase in the proportion of cells in the G_1 phase of the cell cycle (i.e. cause G_1-arrest) of early passage cells. Mouse cells were seeded and cultured in AP + 10% FCS followed by AP + 0.2% BS or AP + 10% FCS as described above. Cultures were harvested 2 days later, stained with DNA fluorochromes, and analysed by flow cytometry (see Section 4.2.2 for details of method). The results of this experiment are displayed in

FIGURE 2.1

Early passage mouse and rat embryo fibroblasts, BHK 21 cells, and HEK cells were cultured in AP + 10% FCS then shifted to AP + 0.2% BS as described in the text. At the time of AP + 0.2% BS addition, and at indicated times, cultures were pulse-labeled for 1 h with 10 μCi/ml [^{3}H] thymidine, then "chased" for 1 h in AP only. Cells were then assayed for DNA synthesis by the method described in Section 2.2.1. On days 4 and 5 mouse and rat cultures were serum stimulated (AP + 10% FCS) and 2 days later they were pulse-labeled as described above.

Symbols:

● Mouse embryo fibroblasts

▲ Rat embryo fibroblasts

■ HEK cells

★ BHK 21 cells

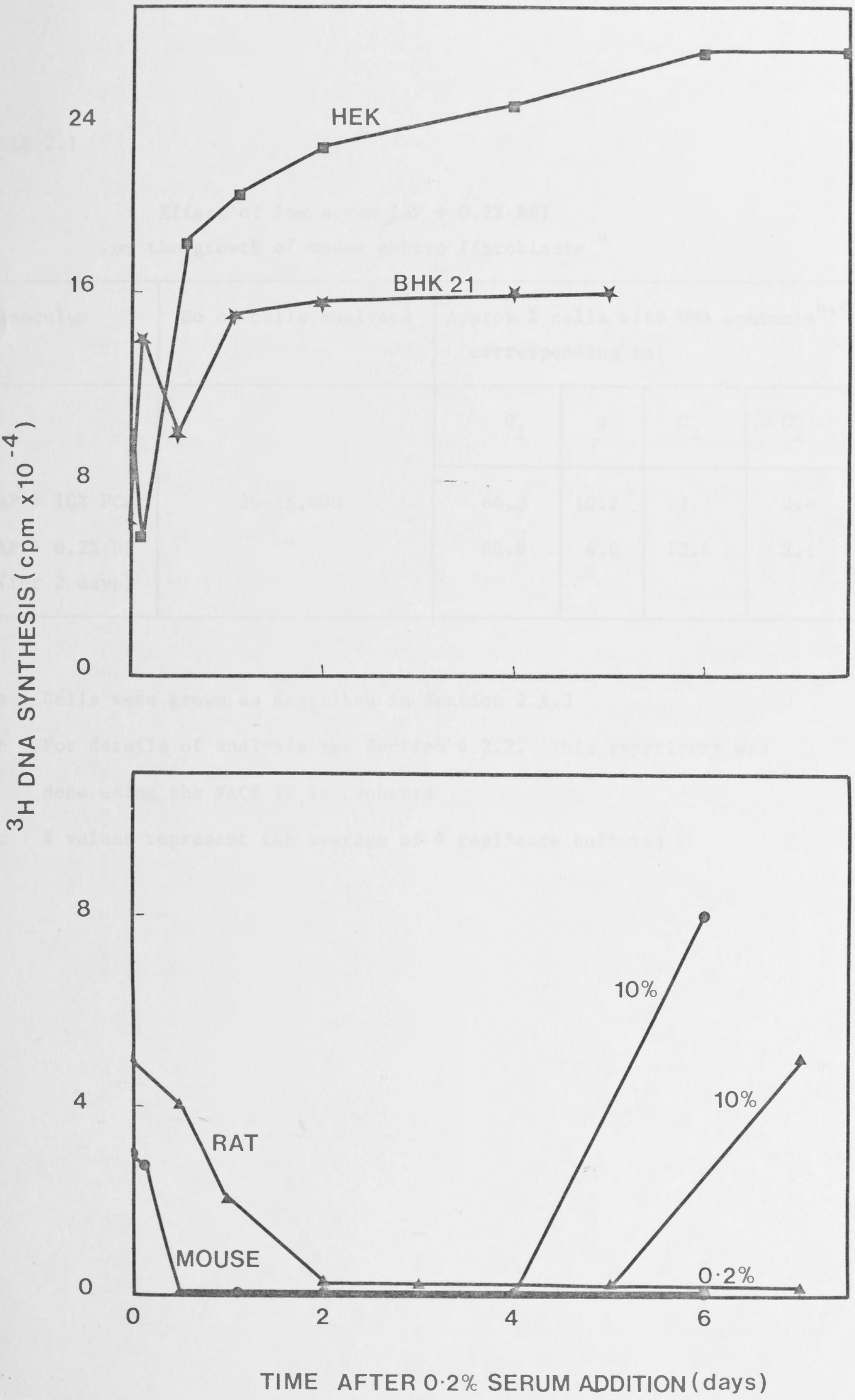

24
16
8
0
HEK
BHK 21
^{3}H DNA SYNTHESIS (cpm x10^{-4})
8
4
0
10%
10%
RAT
MOUSE
0·2%
0
2
4
6
TIME AFTER 0·2% SERUM ADDITION (days)

Table 2.1 and showed that this low serum culturing method did cause a selective increase in G_1 cells (81% in G_1 compared to 64% for growing cells) and was therefore adequate for the stimulation experiments described in this thesis.

2.1.4 INFECTION AND SERUM STIMULATION

(growing or arrested)

Cells were infected with Ad 5 (or mutant viruses) in 1.0 ml of AP without serum for 1.5 hours (h) or with 1.0 ml of AP only for a mock infection. The inoculum was then removed and either (AP+10% FCS)
the original medium replaced (virus infected and mock infected cultures) or AP + 10% FCS was added (serum stimulated cultures). Except where otherwise indicated, in all experiments the multiplicity of infection (m.o.i.) was between 10 iu/cell and 20 iu/cell.

2.1.5 PREPARATION OF RAT BRAINS (see Section 3.3.3)

At appropriate times after pulse-labeling with [^{3}H] thymidine, rats were anesthetised with ether, brains removed, and snap-frozen in liquid N_2. They were then stored at -70^{o}C until sectioning. Brains were sectioned using a freezing microtome (-20^{o}C); sections mounted on glass microscope slides, then fixed in acetone (-20^{o}C for 10 minutes). Slides were then processed for autoradiography or for viral antigen staining as described in Sections 3.2.4 and 2.2.4 respectively.

2.2 BIOCHEMICAL METHODS

2.2.1 DIRECT MEASUREMENT OF INTRACELLULAR DNA SYNTHESIS

For some experiments described in this thesis a simple and rapid technique for measuring DNA synthesis was adopted. After labeling with [^{3}H]thymidine (for indicated times) cultures were washed twice with phosphate-buffered saline (PBS) and then harvested by treatment with 0.025% trypsin in PBS. Cells were suspended in 250 µl of PBS and 80 µl aliquots were removed and spotted on to Whatman 3MM 2.0 cm paper filter disks. Disks were washed twice for 10 minutes in cold 10% trichloracetic acid and twice for 10 minutes in cold ethanol. Filter disks were dried and their radioactivity was measured as described in Section 2.2.2.

2.2.2 DETERMINATION OF RADIOACTIVITY

After processing of filter disks as described above, acid insoluble radioactivity was determined by placing filters in 5 mls of toluene based scintillation fluid (5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter). The radioactivity was then measured in a Packard series 3000 liquid scintillation spectrometer.

When ^{3}H and ^{32}P were counted in the same sample, standards in the same batch of scintillation fluid were also counted. Counts were converted to counts per minute (c.p.m.) and corrected for background and ^{32}P contamination of ^{3}H channel using a PDP 11 computer.

2.2.3 AUTORADIOGRAPHIC MEASUREMENT OF DNA SYNTHESIS

As described in Section 3.2.4.

2.2.4 TESTS FOR VIRAL ANTIGENS

Cells were grown on glass cover slips (diameter, 13mm) in 50mm plastic petri dishes. At appropriate times cover slips were removed, fixed in acetone (-20°C), and tested for viral antigen production by the indirect fluorescent-antibody test using rabbit P antiserum (which reacts primarily with the DNA-binding protein (Russell et al., 1967) ; V antiserum (which reacts with Ad 5 virions); and ALP antiserum (which reacts with early viral proteins other than the DNA-binding protein (see Section 2.2.5).

2.2.5 PREPARATION OF ALP ANTISERUM

Confluent monolayer cultures of rabbit RK 13 cells were infected with approximately 10 iu/cell of ts 125 and treated with 14 µg/ml of cytosine arabinoside. This drug was used to inhibit viral DNA replication thereby preventing the synthesis of late viral antigens as only early antigens were required to stimulate antibody production. Cells were then incubated at the nonpermissive temperature (39.5°C) for ts 125 replication to prevent the synthesis of the adenovirus DNA-binding protein which I also did not want in the antigen preparation. 48 h later, at which time there was little cytopathic effect, cells were harvested by trypsinisation; washed; lysed by freezing and

thawing; and centrifuged at 45,000 rpm for 5 h. The clear supernatant fraction containing viral and cellular proteins was removed by aspiration and stored frozen at -20°C. This extract was later inoculated into rabbits to stimulate antibody production using the schedule outlined by Russell et al.,(1967).

2.2.6 CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ASSAY

As described in Section 5.3.4.1.

2.3 BIOPHYSICAL METHODS

2.3.1 ULTRACENTRIFUGATION OF TOTAL INTRACELLULAR DNA

After lysis of cells and phenol extraction of total intracellular DNA, the techniques of equilibrium gradient centrifugation and analytical ultracentrifugation in neutral CsCl were used to separate Ad 5 and cellular DNA. These techniques are described in detail in Sections 3.2.2 and 3.2.3.

2.3.2 COMBINED AUTORADIOGRAPHY AND MICROSPECTROPHOTOMETRY

As described in Sections 3.2.4 and 3.2.5.

2.3.3 FLOW CYTOMETRY

Details are as described in Section 4.2.2.

2.3.4 PURIFICATION OF [^{32}P]Ad 5 VIRUS AND DNA

Monolayers of human KB cells were infected as described and the virus inoculum was then replaced with AP + 10% FCS for 5 to 6 h at which time this medium was replaced with phosphate free Eagles medium supplemented with 50 µCi/ml of ^{32}P and 1% FCS. Cells were incubated at 37°C from 2 to 4 days until all cells had rounded-up. Cells were then collected and frozen and thawed 3 times in 20mM Tris, pH 7.2. The lysed cells were centrifuged at 2000 rpm for 15 minutes in an MSE bench centrifuge and virus purified from the supernatant fluid by 2 cycles of centrifugation in CsCl.

To prepare purified [^{32}P] Ad 5 DNA, virus was lysed and the DNA phenol extracted as described in Section 3.2.2 for total intracellular DNA. The DNA in the aqueous phase was dialysed against STE buffer. This method was originally described by Younghusband and Bellett (1971).

2.4 REAGENTS

Reagents for DNA extraction:

Lysing solution for DNA extraction: 0.5% SDS (w/v); 0.1 M NaCl, 0.01 M Tris pH 7.9 - 8.0; and 5mM EDTA.

Phenol was redistilled in an atmosphere of N_2 and stored under N_2 in sealed tubes at -12°C.

Phenol buffer for DNA extraction: 0.1 M NaCl; 0.1 M Tris, pH 8.0; and 1mM EDTA. For extraction, this buffer was used to saturate the phenol by mixing with an equal volume of chloroform and isoamylalcohol.

CsCl for equilibrium gradients was of reagent grade quality and purchased from Metallgesellschaft AG, Frankfurt. CsCl for analytical centrifugation was of optical grade quality and purchased from the Sigma Chemical Co.

Enzymes: Protease type VI was purchased from the Sigma Chemical Co. A stock solution (10 mg/ml in distilled H_2O) was incubated at 56°C for 30 minutes prior to use and stored at -20°C. Ribonuclease A was purchased from the Sigma Chemical Co. A stock solution (400 µg/ml in distilled H_2O) was heated at 96°C for 5 minutes to inactivate any contaminating deoxyribonuclease activity and stored in solution at 4°C. Snake venom (Crotalus atrax, Sigma Chemical Co., V-700) containing 5'-nucleotidase activity was stored frozen at -20°C as a 10 mg/ml stock solution in 50mM Tris, pH 7.4. Just prior to use, 100 µl was removed and diluted into 2.5 mls of distilled H_2O and 50 µl of this was used in the reaction mixture for assaying cyclic nucleotide phosphodiesterase activity (see Sections 5.3.4.1 and 5.3.4.2).

Preparation of anion-exchange resin for cyclic nucleotide phosphodiesterase assay: 500 g of anion-exchange resin (Dowex) was dissolved in 2 l of 0.5 N NaOH then filtered and exhaustively washed in distilled H_2O. This step was then repeated. The resin was resuspended in 3 volumes of 0.1 N HCl and left overnight at 4°C. Following this it was washed with 6 l of distilled H_2O then resuspended in 3 volumes of distilled H_2O and stored at 4°C until used.

Stains for Flow Cytometry: Ethidium Bromide purchased from the Sigma Chemical Co. was stored in the dark at 4°C as a stock solution of 250 µg/ml with 1.0% triton-x-100 (v/v). Mithramycin

was purchased from Chas. Pfizer and Co. Inc. and a stock solution (125 µg/ml in 75mM $MgCl_2$ and 1.0% triton-x-100 (v/v)) was stored at 4^oC.

Fluorescein isothiocyanate conjugated sheep-anti-rabbit immunoglobulin was purchased from Silenus Laboratories, Melbourne, and stored in the dark at 4^oC as a stock solution diluted 1/10 in PBS.

Cell Cycle Inhibitors: Cycloheximide and N^6, $O^{2'}$-dibutyryladenosine monophosphoric acid (dbc AMP) were both purchased from the Sigma Chemical Co. A stock solution of cycloheximide (3 µg/ml in distilled H_2O) was stored at 4^oC and stock dbc AMP (100mM in distilled H_2O) was stored frozen at -20^oC. Cytosine arabinoside, used in the preparation of ALP antiserum, was purchased from the Sigma Chemical Co. and stored in crystalline form at 4^oC. This was added as a solid to infected cultures during preparation of the extract to use as antigen for stimulation of antibody production.

Radioisotopes: ^{32}P (Carrier-free) was obtained from the Australian Atomic Energy Commission, Sydney. [methyl-^3H] thymidine ([^3H]thymidine) (48 Ci/mmole) and [2,8] -^3H adenosine 3', 5'-cyclic phosphate (30-50 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, U.K.

All reagents and chemicals referred to in this thesis and not specifically listed above were of Analar grade and all obtained from British Drug Houses, U.K.

CHAPTER THREE

CELLULAR DNA REPLICATION IN Ad 5 INFECTED, QUIESCENT RODENT CELLS

3.1 INTRODUCTION

Lack of sensitivity to growth-arresting conditions is a feature commonly observed of in vitro-transformed cells. One particularly interesting phenomenon which would seem to be related to this is the property of several DNA viruses to stimulate the synthesis of cellular DNA, even in cells which are density-inhibited or quiescent (G_1- arrested) (see review by Black, 1968). Several serotypes of the human adenoviruses, for example, have been shown to induce cellular DNA synthesis in both density-inhibited (Shimojo and Yamashita, 1968) and arrested hamster and mouse cells (Strohl, 1969; Younghusband et al., 1979). In addition, SV40 (Gershey, 1979) and Rous Sarcoma virus (RSV) (Kobayashi and Kaji, 1978) will induce cellular DNA synthesis in non-cycling cells. Although the phenomenon has been frequently reported in the literature, the mechanism by which this induction occurs is not understood, but it is likely that in some way viruses alter cell cycle controls.

Early experiments from Strohl's laboratory (Strohl, 1969) provided the first clues as to the viral contributions to the induction of cellular DNA synthesis. Strohl used Ad 12 infected G_1-arrested hamster cells in order to study Ad 12 induced cellular DNA replication. This system was an advantageous one to use as Ad 12 had been convincingly shown not to replicate viral DNA in these cells (Doerfler, 1969). Strohl's experiments showed that Ad 12 did indeed stimulate cellular DNA synthesis (as

measured by autoradiography) which was subsequently followed by a wave of mitoses. This study also showed that DNA synthesis occurred only in Ad 12 T antigen positive cells and therefore suggested that the induction of cellular DNA synthesis required viral gene expression, and probably expression of a gene or genes from one or more of the viral early regions (see Chapter One).

Subsequent studies in which productive versus non-productive infections were compared, indicated, however, that the controls of adenovirus induced cellular DNA replication were probably complex. Laughlin and Strohl (1976a,b) showed that the induction of DNA synthesis in quiescent hamster cells by Ad 12 and serum was inhibited by 0.03 µg/ml actinomycin D (AMD), whereas the induction was refractory to AMD when Ad 2 was used as the initiating agent. Ad 2 DNA does replicate in hamster cells. Under the same conditions, viral DNA replication was inhibited considerably and virus yield was reduced >90% of control levels (Laughlin and Strohl, 1976b), but T antigen synthesis continued unaffected by the presence of the drug.

Similarly, Ad 12 T antigen synthesis was unaffected by AMD, although RNA synthesis was reduced 98% (Laughlin and Strohl, 1976a). This seemed likely to be a selective inhibition of rRNA synthesis as this concentration of AMD in these cells does not affect mRNA synthesis (Martin and Brown, 1967). By timed addition of drug to serum stimulated cells it was found that the AMD sensitive point was 5 hours before the onset of cellular DNA synthesis. Results from these experiments suggested

that (i) Induction of DNA synthesis by Ad 2 and Ad 12 probably occurs through different mechanisms; (ii) For the nonpermissive infection, the induction requires an AMD-sensitive step, which might be the induction of rRNA synthesis. This AMD-sensitive step is apparently not required for induction in a permissive infection, and (iii) Probably virus early gene expression is required.

Also about 1976, Minekawa and colleagues were investigating the relationships between transformation and cellular and viral DNA synthesis using some host-range (hr) and temperature-sensitive (ts) mutants of Ad 5 they had isolated (Minekawa et al., 1976). They found one class of hr mutants which was early region defective and therefore did not synthesise viral DNA, but was positive for the induction of hamster cell DNA synthesis and transformation. This result strongly suggested that early viral gene expression was required for induction of cellular DNA synthesis and was thus confirmatory of the results from Strohl's laboratory for the nonpermissive infection (see earlier). Minekawa et al. (1976) also isolated two classes of ts mutants which are of interest. Both classes are early region mutants and both were shown to be defective for viral DNA replication but positive for induction of cellular DNA replication. One of these two classes of mutants (their class I), was defective for hamster cell transformation, whereas the other was positive. The early region mutants which were transformation defective were less potent at inducing cellular DNA synthesis than the transformation positive mutants. This result suggests some

relationship between adenovirus induced cellular DNA replication and cellular transformation, which seems a potentially interesting area to examine. It would be extremely valuable to compare these mutants with others which have been better characterised (see below) in terms of the relationships among viral DNA replication, virus induced cellular DNA replication, and transformation. No such comparisons have been reported and I have been unable to obtain any of these adenovirus mutants.

Another class of ts mutants of Ad 5, which showed different properties with respect to rodent cell transformation (Ginsberg et al., 1974; Williams et al., 1974), has been isolated (Williams et al., 1974). These mutants are reasonably well-characterised and the position of the mutations have been identified as well as the regions of the adenovirus genome responsible for transformation. The regions which affect transformation are the left hand 1-7.3% of the Ad 5 genome (Graham et al., 1977); the region coding for the DNA-binding protein (62-68%) (Ginsberg et al., 1974); and the region coding for the gene N product (19.8-23.5%) (Williams et al., 1974; Galos et al., 1979). Ts mutants in gene N (ts 36 and ts 37) are defective for viral DNA replication in human cells and have a reduced ability to transform rat cells at 38.5°C, but behave like wild-type virus at 32.5°C (Williams et al., 1974). Mutant ts 125 is defective in viral DNA replication because of a defect in the DNA-binding protein at 38.5°C (Van der Vliet and Sussenbach, 1975) which has recently been localised by sequencing studies to a single nucleotide base change in the DNA coding for the

carboxy region of the DNA binding protein (Kruijer et al., 1981). This mutant transforms rat cells with a higher frequency than wild-type Ad 5 at all temperatures (Ginsberg et al., 1974).

Deletion (dl) mutants of Ad 5 which have defective transforming abilities in rodent cells (Jones and Shenk, 1979a; Shenk et al., 1979) have also been isolated (Jones and Shenk, 1979a). Mutants dl 312 and dl 313 are deleted in early regions 1A and 1B respectively, although the deletions do overlap to a small extent (Jones and Shenk, 1979a). They grow on the 293 cell line which is a human cell line transformed with the left hand 11% of the Ad 5 genome (Graham et al., 1977). This portion of the viral genome is expressed and therefore complements the mutant defects. The mutants are defective for growth on untransformed human cells at low multiplicities of infection (Shenk et al., 1979). Until very recently (see below) neither the ts mutants isolated in Williams' laboratory (Williams et al., 1974) nor the dl mutants had been studied with respect to their effects on cellular growth controls. This seemed like a potentially interesting area to investigate, particularly as a result of the observations from Minekawa's laboratory (Minekawa et al., 1976). Some degree of correlation between transformation frequency and the induction of cellular DNA synthesis has also been suggested for SV40 (Butel and Soule, 1978; Gershey, 1979; Lehman and Defendi, 1970; Todaro and Green, 1966). In this connection, SV40 T antigen which has been implicated in transformation (Butel and Soule, 1978; Fluck and Benjamin, 1979) has been clearly shown to induce cellular DNA replication (Butel and Soule, 1978; Graessmann et al., 1980).

In this chapter, I have investigated the controls of Ad 5 induced cellular DNA synthesis using the well-characterised ts and d1 mutants described above. At the time this work was done, no such studies had been reported, but very recently both classes of mutants have been tested by two other groups (Rossini et al., 1981; Shiroki et al., 1981). These studies are discussed in Section 3.4.

Using this approach, I was able to investigate the specific virus gene requirements for the induction and to establish clearly whether or not the induction is early region controlled. In addition, I have used an inhibitor of protein synthesis to investigate whether any cellular gene products are also involved and cell cycle analysis to determine whether a complete cell cycle is induced by Ad 5 infection.

3.2 METHODS OF MEASURING DNA SYNTHESIS

The most convenient means of assaying for increases in cellular DNA synthesis by treatment with various mitogenic agents are autoradiography and the direct measurement of radioactivity incorporated from [^{3}H] thymidine into trichloracetic acid-precipitable material from lysates. Both these methods are rapid but have the disadvantage that if the mitogenic agent happens to be a DNA virus, such as is studied in this thesis, it is not possible to distinguish between radioactivity incorporated into cellular DNA and that incorporated into viral DNA. Thus, whilst these methods serve as useful indicators of the mitogenic ability of a DNA virus, they do not provide a clear enough result alone. Unless they are accompanied by analysis

which separates radioactivity incorporated into viral from cellular DNA, the use of such methods is limited. As a result, this led me to investigate two alternative approaches to study the mitogenic ability of Ad 5. Both these methods utilise ultracentrifugation.

3.2.1. HIRT SUPERNATANT METHOD

The first method I investigated was based on the observations of Hirt (1967) who noticed that 1M NaCl at $4^{o}C$ selectively precipitated cellular DNA from polyoma virus infected cells after lysis with protease and SDS. After centrifugation at 17,000 g for 30 min, the cellular DNA was essentially sedimented along with the SDS, whilst the polyoma DNA remained in the supernatant fraction. In view of these results, the Hirt supernatant method was attempted in order to separate cellular DNA from Ad 5 DNA.

HEK cells were infected with Ad 5 or mock infected and 16 h later they were pulse-labeled for 2 h with 10 µCi/ml [^{3}H] thymidine. This was followed by a 2 h "chase" at which time the cells were lysed and treated as described by Hirt (1967). [^{32}P] Ad 5 DNA was then added to the lysate. Results from this experiment (Table 3.1) illustrated that [^{3}H] radioactivity is distributed evenly between the supernatant and the pellet. Thus, unlike the results obtained by Hirt (1967) for polyoma infected cells, this method is a poor means of measuring the relative amounts of Ad 5 and cellular DNA synthesis in infected cells. That is, it would not be possible to decide whether an apparent stimulation of DNA synthesis was due to viral or cellular DNA synthesis or both.

TABLE 3.1

Hirt Separation of Ad 5 and Cellular DNA from infected HEK cells *

Inoculum	Fraction	Relative [^{3}H] thymidine distribution (% cpm)	Relative [^{32}P] Ad 5 DNA distribution (% cpm)
MOCK	Supernatant	11.8	82.9
	pellet	88.2	17.1
Ad 5	Supernatant	52.8	76.1
	pellet	47.6	23.9

* The experiment illustrated in Table 3.1 is representative of several

Cells were infected and labeled as described in Section 2.1.4 and the text. After labeling, cells were lysed as described by Hirt (1967), [^{32}P] Ad 5 DNA added to the lysate, and the samples were incubated at 4^{o}C overnight. They were then centrifuged and aliquots of the supernatant and pellet fractions were removed, spotted on to filter disks, and the radioactivity determined. The experiment was carried out in triplicate.

3.2.2 EQUILIBRIUM GRADIENT CENTRIFUGATION

As the method of Hirt (1967) proved unsatisfactory for my purposes, I decided to try equilibrium gradient centrifugation to separate Ad 5 and cellular DNA. The method of Tyndall et al (1978) was adopted and the details are described below.

HEK cells were once again infected with Ad 5 or mock infected and labeled with [^{3}H] thymidine. At 24 h after infection cultures were washed with phosphate-buffered saline (PBS) and lysed by incubation at 37^{o}C with protease VI in lysing solution (see section 2.4 for reagents). DNA was twice extracted with an equal volume of aqueous buffer-saturated phenol and a mixture of chloroform and isoamylalcohol (24:1) and then dialysed against STE buffer (for details of reagents see section 2.4). Following this procedure each DNA sample was mixed with [^{32}P] Ad 5 DNA, STE, 0.1% sarkosyl, and CsCl to give a final volume of 8 ml and a density of 1.704 to 1.707 g/ml. The samples were over-laid with paraffin oil and centrifuged to equilibrium in a 50 Ti-rotor at 33,000 rpm for 40 h at 20^{o}C. The gradients so formed were fractionated from the bottom and 80 µl aliquots were spotted on to Whatman 3MM 2.0 cm paper filter disks. These were washed and the radioactivity determined using a Packard scintillation spectrometer. The curves of total thymidine labeled DNA were analysed into virus DNA and cell DNA components using a PDP 11 computer and principles described by Younghusband et al (1979).

Results of an experiment using this method of measuring DNA synthesis and computer analysis of the DNA profiles illustrated that the procedure was capable of giving accurate separation of radioactivity incorporated into cellular and Ad 5 DNA (see Fig. 3.1). This method of DNA analysis was therefore adopted for studying Ad 5 induced DNA synthesis in this thesis.

3.2.3 ANALYTICAL ULTRACENTRIFUGATION

For analysis of total intracellular DNA into viral and cellular components by Model E ultracentrifugation, phenol extracted DNA was treated first with ribonuclease A (20 µg/ml for 1 h at 37°C) and then with protease VI (0.3 mg/ml for 1 hr at 37°C). Following this procedure, DNA was reextracted twice with phenol (see above) and dialysed against STE buffer. DNA was ethanol precipitated (-20°C for 2 h); sedimented at 10,000 rpm for 20 minutes; then redissolved in 0.1 x STE buffer. 2-3 µg of DNA was added to 1.0 ml of neutral CsCl (density 1.704 to 1.707 g/ml) and centrifuged in an automated Beckman Model E Ultracentrifuge. The DNA profiles so formed were then measured by absorbance in the ultraviolet region at 260nm. In some cases Micrococcus lysodeikticus DNA was added as a density standard prior to centrifugation. The area under the viral and cellular DNA curves was estimated using the curve fitting program developed by Reisner (1980).

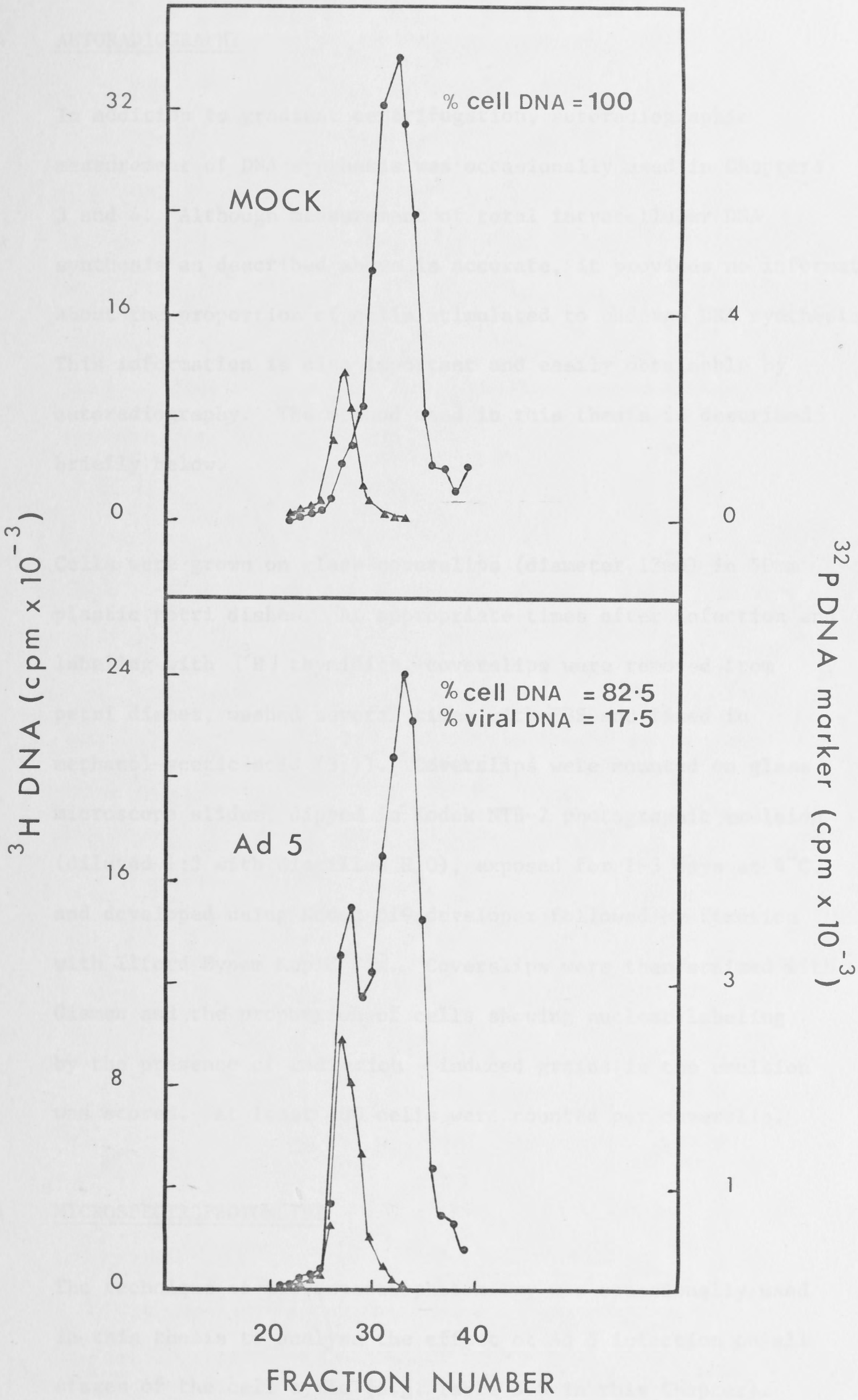
FIGURE 3.1

Separation of cellular and viral DNA by equilibrium gradient centrifugation and computer analysis. HEK cells (approx. 3×10^6 cells /50 mm dish) were infected with 10 iu/cell or mock infected as described in Section 2.1.4. Cells were labeled with [^{3}H] thymidine from 13-24 h post infection at which time they were lysed, the DNA extracted, and centrifuged to equilibrium as described in Section 3.2.2. Both panels represent equilibrium gradient profiles of labeled DNA. The inserts represent the estimated proportion of [^{3}H] radioactivity in cellular and viral DNA, relative to a third uninfected cell control run at the same time, and analysed according to the method of Younghusband et al., (1979).

Symbols:

● [^{3}H] thymidine labeled intracellular DNA

▲ [^{32}P] Ad 5 DNA marker


MOCK
% cell DNA = 100
Ad 5
% cell DNA = 82·5
% viral DNA = 17·5
32
16
0
24
16
8
0
4
0
3
1
20
30
40
FRACTION NUMBER
^{3}H DNA (cpm x 10^{-3})
^{32}P DNA marker (cpm x 10^{-3})

3.2.4 AUTORADIOGRAPHY

In addition to gradient centrifugation, autoradiographic measurement of DNA synthesis was occasionally used in Chapters 3 and 4. Although measurement of total intracellular DNA synthesis as described above is accurate, it provides no information about the proportion of cells stimulated to undergo DNA synthesis. This information is also important and easily obtainable by autoradiography. The method used in this thesis is described briefly below.

Cells were grown on glass coverslips (diameter 13mm) in 50mm plastic petri dishes. At appropriate times after infection and labeling with [^{3}H] thymidine, coverslips were removed from petri dishes, washed several times with PBS and fixed in methanol-acetic acid (3:1). Coverslips were mounted on glass microscope slides, dipped in Kodak NTB-2 photographic emulsion (diluted 1:3 with distilled H_2O), exposed for 1-3 days at 4^oC, and developed using Kodak D19 developer followed by fixation with Ilford Hypam Rapid Fix. Coverslips were then stained with Giemsa and the proportion of cells showing nuclear labeling by the presence of radiation - induced grains in the emulsion was scored. At least 400 cells were counted per coverslip.

3.2.5 MICROSPECTROPHOTOMETRY

The technique of microspectrophotometry was occasionally used in this thesis to analyse the effect of Ad 5 infection on all stages of the cell cycle (e.g. Table 3.8 in this Chapter).

Cells were seeded on to glass microscope slides (3.5×10^5 cells/slide) and placed in 10cm glass petri dishes. At indicated times after infection slides were washed in PBS and fixed in 3:1 methanol/acetic acid. Slides for staining were then placed in distilled H_2O to rehydrate the cells, hydrolysed for 15 minutes in 1N HCl (60^o) and stained with Feulgen.

Integrated absorbance of stained nuclei was measured at a wavelength of 565nm using a Zeiss 02 photometer system interfaced to a digital PDP 12 computer. The system was operated in conjunction with the APAMOS (automatic photometric analysis of microscopic objects by scanning) MODIFIED program, described by Gould (1979).

3.3 RESULTS

3.3.1 INDUCTION OF CELLULAR DNA SYNTHESIS BY Ad 5

Rat and mouse fibroblasts have been shown to be semipermissive for replication of the "nononcogenic" group C human adenoviruses, the group which includes Ad 5 (Gallimore, 1974; Younghusband et al., 1979). In these cells, the production of virus particles is markedly reduced compared to a permissive infection (e.g. in mouse fibroblasts only 0.2 iu/cell was observed (Younghusband et al., 1979)), late antigen expression is reduced, and viral DNA replication is reduced. However, early viral antigen expression occurs at a high level in both cell types. Thus these cells seemed like a useful cell system for studying the induction of cellular DNA synthesis with adenovirus

mutants. Furthermore, since these mutants had been previously screened for transforming ability in rat cells (Ginsberg et al., 1974; Jones and Shenk, 1979a) it seemed like an advantageous way of more directly comparing induction and transforming capacities of the mutants.

The first experiment carried out was to determine whether Ad 5 and fresh serum growth factors were able to stimulate cellular DNA synthesis in G_1-arrested rat fibroblasts (not previously reported); BHK 21 cells (reported by Laughlin and Strohl (1976b); Harris and Strohl (1980) for Ad ~~2~~); mouse fibroblasts (reported by Younghusband et al., 1979); and the permissive human cell line HEK. The HEK cell line was G_1-arrested by growing the cells to confluency, as they could not be arrested in low serum medium (see Fig. 2.1). Otherwise, cells were infected, labeled and analysed as described above.

A clear stimulation of cellular DNA synthesis was observed in all serum treated and Ad 5 infected cells (Fig. 3.2 (mouse and rat); Fig 3.3 (HEK)), excepting BHK 21 cells (Fig. 3.4). In this case addition of fresh serum induced DNA synthesis, but infection with Ad 5 at either a low (15 iu/cell) or high (700 iu/cell) multiplicity of infection failed to induce any cellular or viral DNA synthesis. This lack of induction by Ad 5 is not due to a lack of infection as Ad 5 is able to induce the enzyme thymidine kinase in these cells (B.F. Cheetham, personal communication). The result with BHK 21 cells is in contrast to previous reports with Ad 2 (see above) and is probably because continuous culturing of this cell line has

FIGURE 3.2

Induction of cellular DNA synthesis by Ad 5 and serum in rat and mouse cells. Rat or mouse cells arrested in G_1 by incubation in 0.2% serum were infected with Ad 5, mock infected, or treated with 10% FCS. All panels represent equilibrium gradient profiles of [^{3}H] thymidine labeled DNA extracted as described in Section 3.2.2. Cells were labeled 12 to 24 h (rat) and 24 to 48 h (mouse) post infection.

Symbols: ● [^{3}H] thymidine labeled intracellular DNA

■ [^{32}P] labeled Ad 5 DNA marker

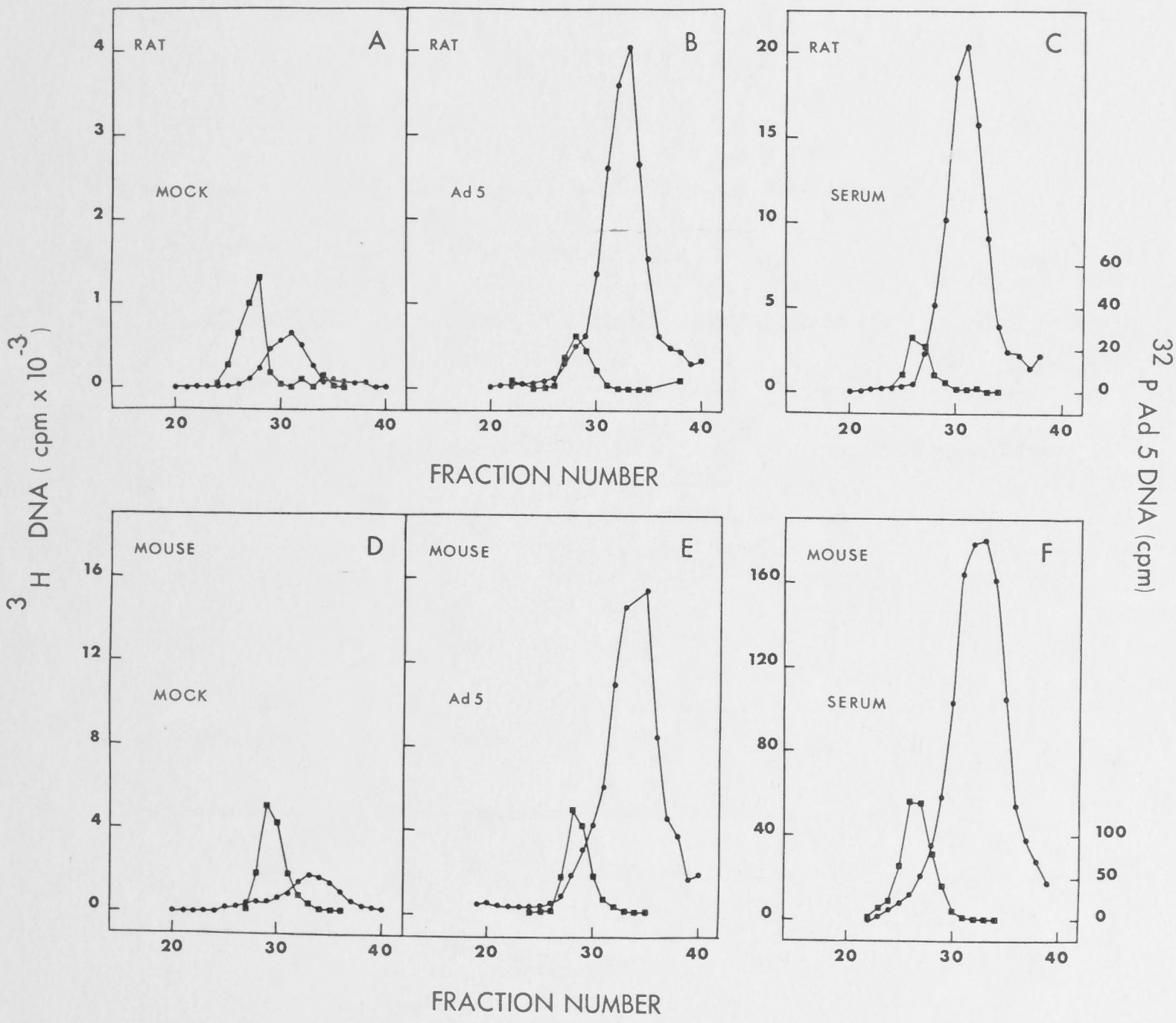

RAT
A
MOCK
RAT
B
Ad 5
RAT
C
SERUM
MOUSE
D
MOCK
MOUSE
E
Ad 5
MOUSE
F
SERUM
FRACTION NUMBER
FRACTION NUMBER
^{3}H DNA (cpm x 10^{-3})
^{32}P Ad 5 DNA (cpm)

FIGURE 3.3

Induction of cellular DNA synthesis by Ad 5 and serum in HEK cells. HEK cells were grown to confluency (usually about 6 to 8 days in 10% FCS), then infected with Ad 5, mock infected, or serum treated. All panels represent equilibrium gradient profiles of [^{3}H] thymidine labeled DNA extracted as described in Section 3.2.2. Cells were labeled in this case early after infection (6 to 13 h) as the cell line is permissive for virus replication.

Symbols:

● [^{3}H] thymidine labeled intracellular DNA

▲ [^{32}P] labeled Ad 5 DNA marker

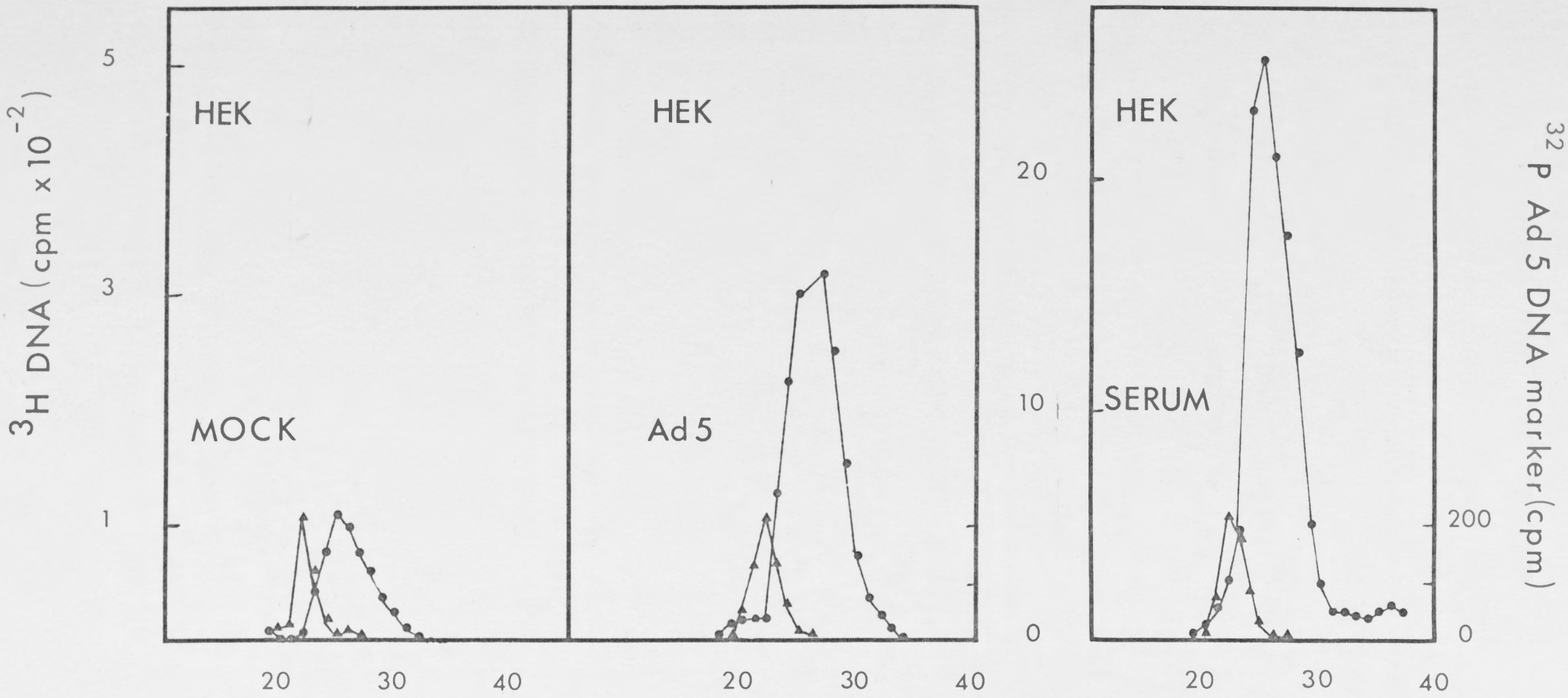

HEK
MOCK
HEK
Ad 5
HEK
SERUM
^{3}H DNA (cpm x10^{-2})
^{32}P Ad 5 DNA marker (cpm)
5
3
1
20
10
0
200
0
20
30
40
20
30
40
20
30
40
FRACTION NUMBER

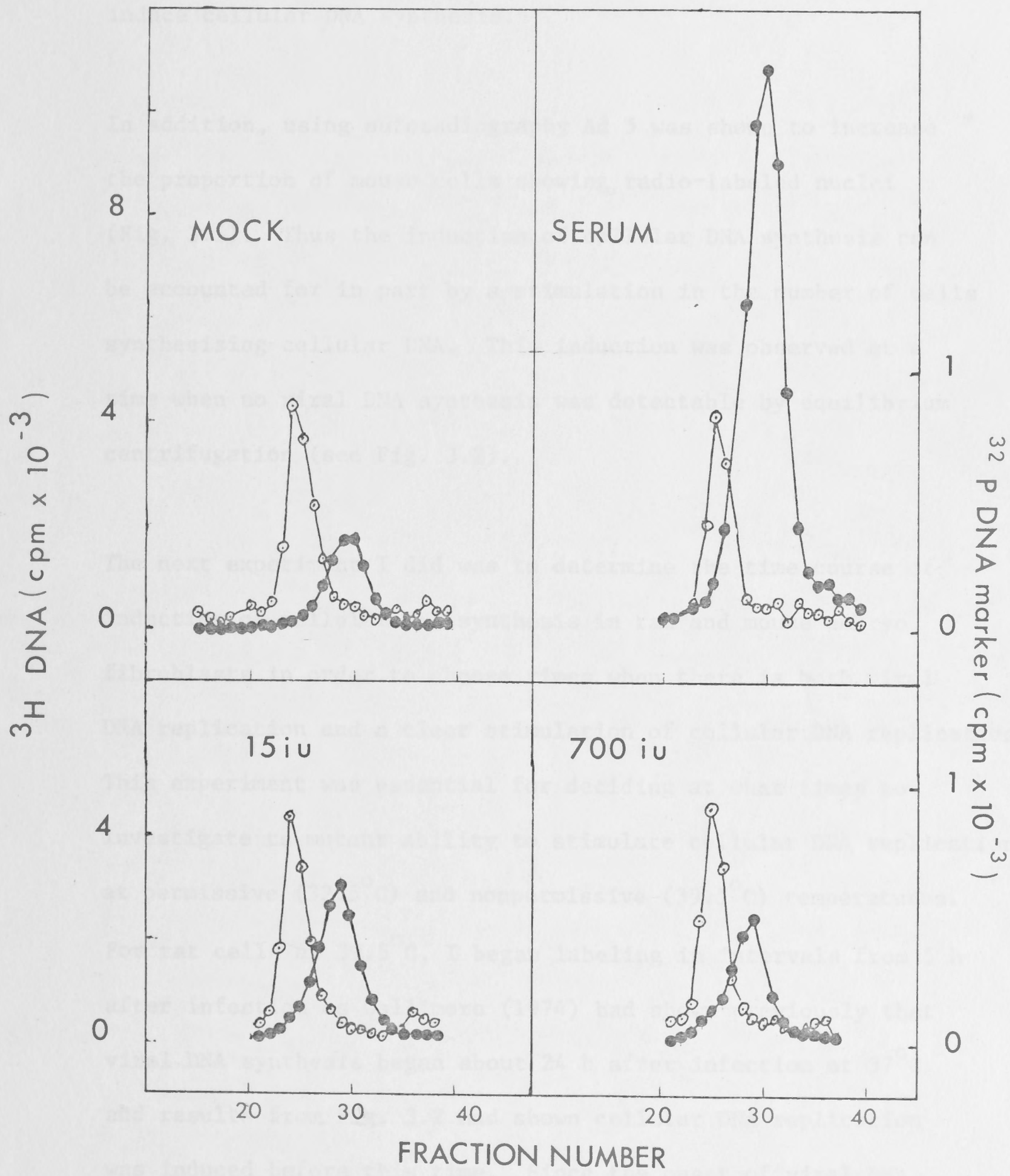
FIGURE 3.4

Induction of cellular DNA synthesis in BHK 21 cells by Ad 5 and serum. Details are as described in the legend to Fig. 3.2. In this case the labeling time used is 8 to 20 h after infection as reported in Harris and Strohl (1980).

Symbols: ● [^{3}H] thymidine labeled intracellular DNA

○ [^{32}P] labeled Ad 5 DNA marker

iu infectious units/cell


MOCK
SERUM
15 iu
700 iu
8
4
0
1
0
4
0
1
0
20
30
40
20
30
40
FRACTION NUMBER
^{3}H DNA (cpm x 10^{-3})
^{32}P DNA marker (cpm x 10^{-3})

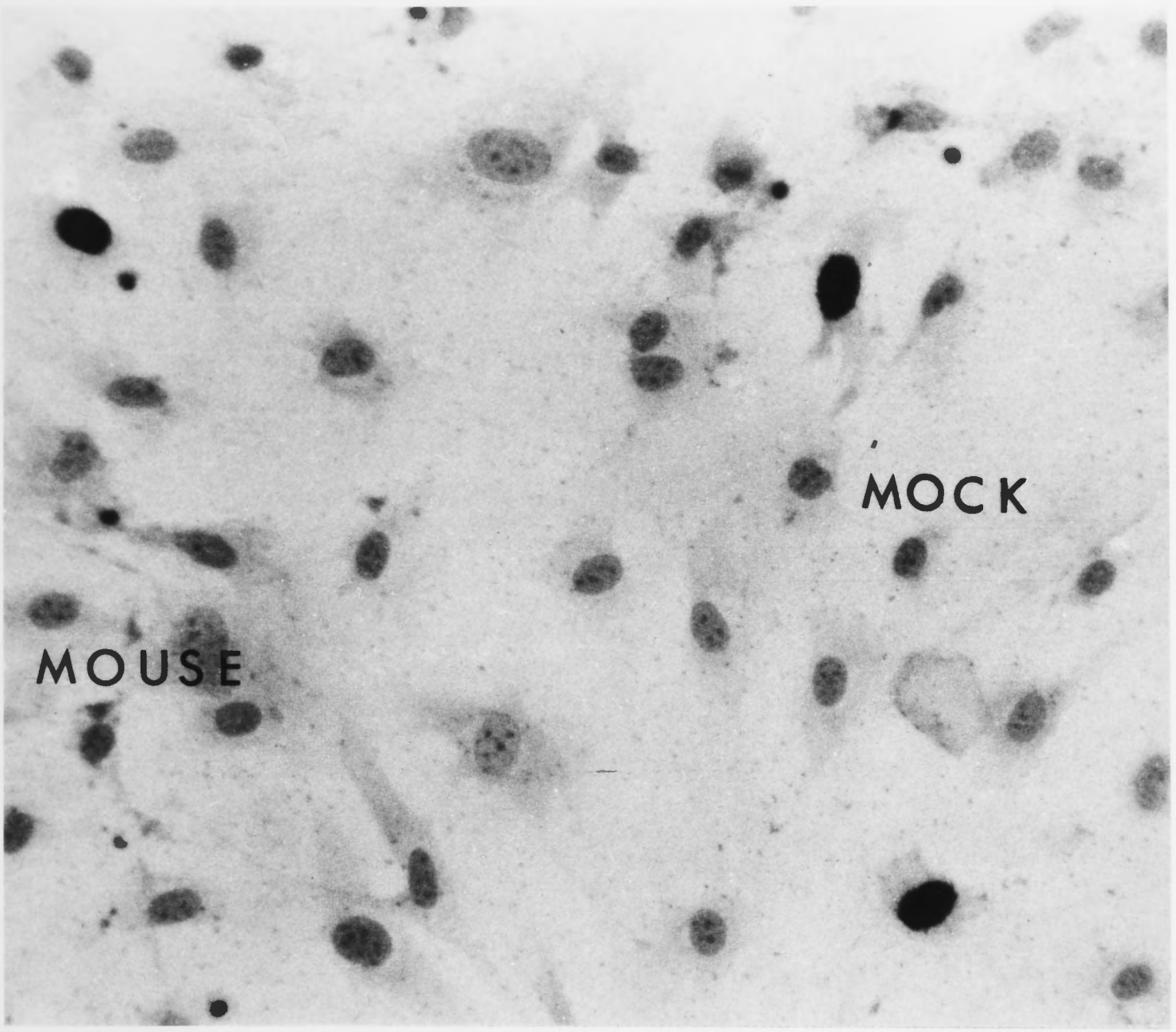
altered its properties with respect to host-adenovirus interactions. This negative result also suggests that there is probably a cellular component required in order for Ad 5 to induce cellular DNA synthesis.

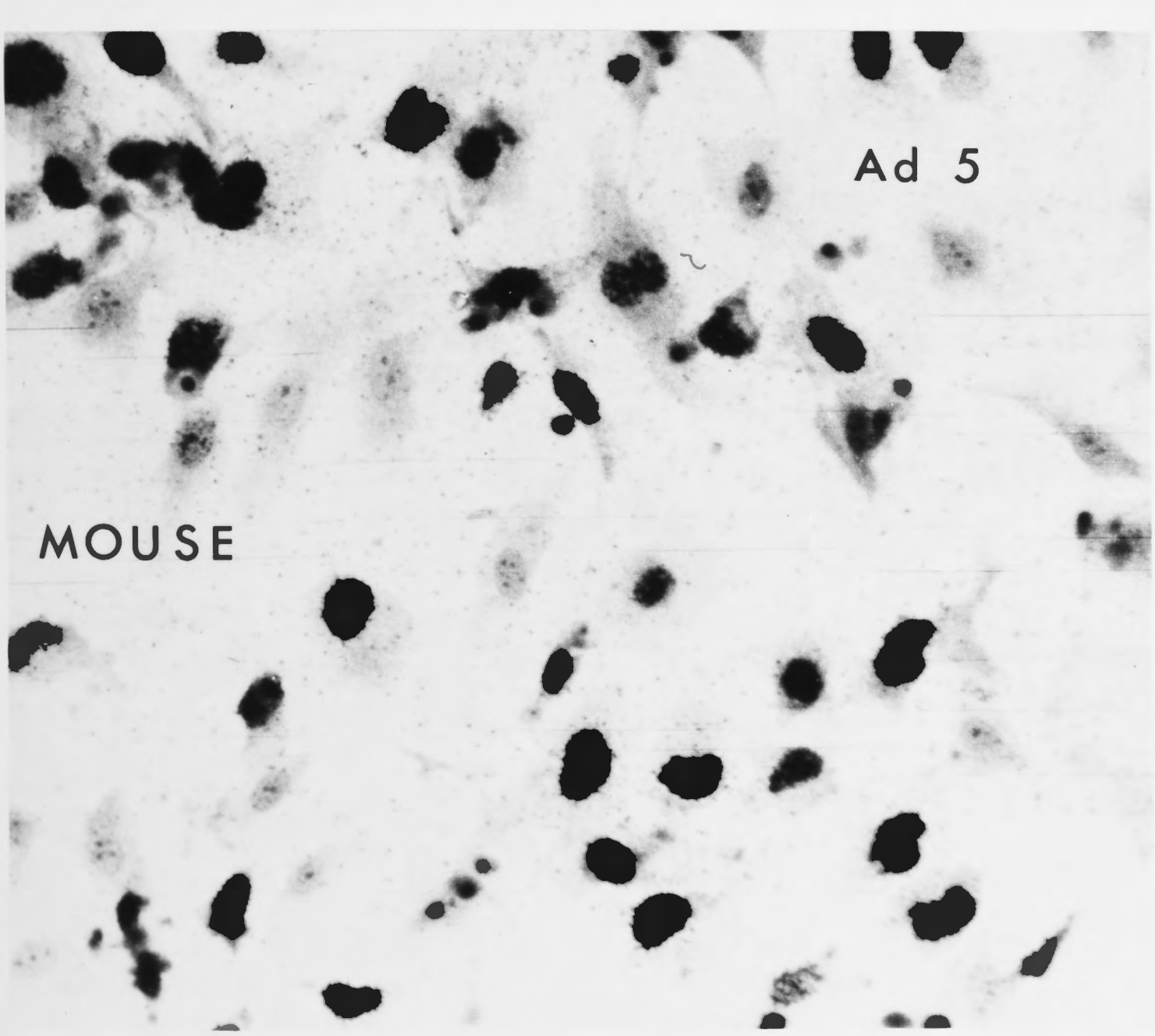
In addition, using autoradiography Ad 5 was shown to increase the proportion of mouse cells showing radio-labeled nuclei (Fig. 3.5). Thus the induction of cellular DNA synthesis can be accounted for in part by a stimulation in the number of cells synthesising cellular DNA. This induction was observed at a time when no viral DNA synthesis was detectable by equilibrium centrifugation (see Fig. 3.2).

The next experiment I did was to determine the time course of induction of cellular DNA synthesis in rat and mouse embryo fibroblasts in order to choose times when there is both viral DNA replication and a clear stimulation of cellular DNA replication. This experiment was essential for deciding at what times to investigate ts mutant ability to stimulate cellular DNA replication at permissive (32.5^{o}C) and nonpermissive (39.5^{o}C) temperatures. For rat cells at 39.5^{o}C, I began labeling in intervals from 5 h after infection as Gallimore (1974) had shown previously that viral DNA synthesis began about 24 h after infection at 37^{o}C and results from Fig. 3.2 had shown cellular DNA replication was induced before this time. Since the onset of viral DNA synthesis is delayed at 32.5^{o}C compared to 37^{o}C (A. Bellett, personal communication) labeling at 32.5^{o}C was begun at 20 h after infection. For mouse cells at 36.5^{o}C, I had no previous

FIGURE 3.5

Stimulation of mouse cells to enter DNA synthesis by Ad 5 infection. Cells were arrested in G_1 by incubation in 0.2% serum for 2 days at which time they were mock infected or infected with Ad 5. They were labeled with [^{3}H] thymidine from 24 to 48 h after infection then coverslips were removed from culture dishes, fixed, and processed for autoradiography (see Section 3.2.4).


MOCK
MOUSE


Ad 5
MOUSE

times from the literature to indicate when to look for viral and cellular DNA replication, so in this case, cells were labeled in 25 h intervals from the time of infection up to 4 days after infection.

Results from this experiment (see Table 3.2) showed that the peak induction of cellular DNA synthesis occurred in both cell types at 39.5°C (rat) and 36.5°C (mouse) between 1 and 2 days after infection, and large amounts of viral DNA were detectable between 1 and 3 days after infection, which peaked in mouse cells at 36.5°C between 2 and 3 days after infection. The onset of viral and cellular DNA replication in rat cells at 32.5°C was delayed as expected with high levels of both types of DNA replication occurring between 44 and 68 h after infection. For ts mutant experiments therefore I decided to test for induction of cellular DNA replication between 1 and 3 days after infection at which time both cellular DNA replication (at 39.5°C and 32.5°C) and viral DNA replication were occurring. For other experiments involving Ad 5 wild-type only at 36.5°C (see Chapters 5 and 6 in particular) in most experiments labeling with [^{3}H] thymidine was done between 1 and 2 days after infection.

TABLE 3.2

Time Course of Induction of Cellular and Viral DNA Synthesis in Ad 5 infected G_1-arrested [a] mouse and rat cells

Conditions	Inoculum	Time of labeling (h)	Viral DNA [b] Component (cpm)	Cellular DNA [b] Component (cpm)	Degree of stimulation [c]
Rat [d], 39.5°C (nonpermissive for ts mutant replication)	MOCK	5-20	0	3346	1.0
	Ad 5	"	699	9937	2.97
	MOCK	20-35	0	4143	1.0
	Ad 5	"	5457	19433	4.69
	MOCK	35-50	0	2048	1.0
	Ad 5	"	2131	3093	1.51
Rat [d], 32.5°C (permissive for ts mutant replication)	MOCK	20-44	0	11995	1.0
	Ad 5	"	2174	29627	2.47
	MOCK	44-68	0	5992	1.0
	Ad 5	"	8824	26844	4.49
Mouse [d], 36.5°C (normal temp. for wild-type Ad 5 replication)	MOCK	0-25	0	18117	1.0
	Ad 5	0-25	781	23820	1.31
	Ad 5	25-50	3804	165746	9.15
	Ad 5	50-75	23092	69016	3.81
	Ad 5	75-100	24162	56777	3.13

a Cells were arrested and infected as described in Chapter 2

b Total [^{3}H] thymidine labeled intracellular DNA from CsCl gradients was analysed into viral and cellular DNA components (see Section 3.2.2)

c Radioactivity in cellular DNA component divided by radioactivity from mock infected cultures in the same fractions

d Mouse and rat cells tested in separate experiments

3.3.2 DEPENDENCE OF THE INDUCTION OF CELLULAR DNA SYNTHESIS ON ADENOVIRUS GENE EXPRESSION

Having established the basic phenomenon of induction of cellular DNA synthesis by Ad 5, using the conditions and cells described above (excepting BHK), and also having demonstrated that the induction is not confined to permissive or semipermissive cells, the next experiments were designed to eliminate the possibility that the phenomenon is simply a nonspecific effect of the inoculation procedure. This was examined in three different ways: (1) Ad 5 was inactivated with ultraviolet (UV) irradiation, or treated with an antiserum to the virus particle; (2) cells were infected with different amounts of an Ad 5 inoculum, and (3) the induction of cellular DNA synthesis by Ad 5 was measured in medium which was completely free of any serum growth factors.

For these experiments, rat or mouse cells were infected with Ad 5 and labeled with [^{3}H] thymidine for indicated time periods. Total intracellular DNA was extracted, centrifuged to equilibrium, and the [^{3}H] radioactivity was analysed by computer into viral and cellular components as described above.

Results from these experiments showed (i) that the induction of cellular DNA synthesis by Ad 5 is prevented by both UV irradiation and antiserum treatment (Fig. 3.6); (ii) the induction varies with the amount of infectious virus (Table 3.3); and (iii) it occurs in the total absence of serum (Fig. 3.7).

FIGURE 3.6

Failure to induce cellular DNA synthesis in Ad 5 infected rat cells after UV irradiation or antibody treatment of the inoculum. The procedure was as outlined in the legend to Fig. 3.2. (A) mock infected; (B) Ad 5 infected control; (C) Ad 5, preincubated with antiserum (1:5) to Ad 5 at 37°C for 0.5 h; (D) Ad 5 pretreated with UV irradiation (800 μWcm^{-2} at 0°C for 0.5 h)

Symbols: ● [^{3}H] thymidine labeled intracellular DNA

■ [^{32}P] labeled Ad 5 DNA marker

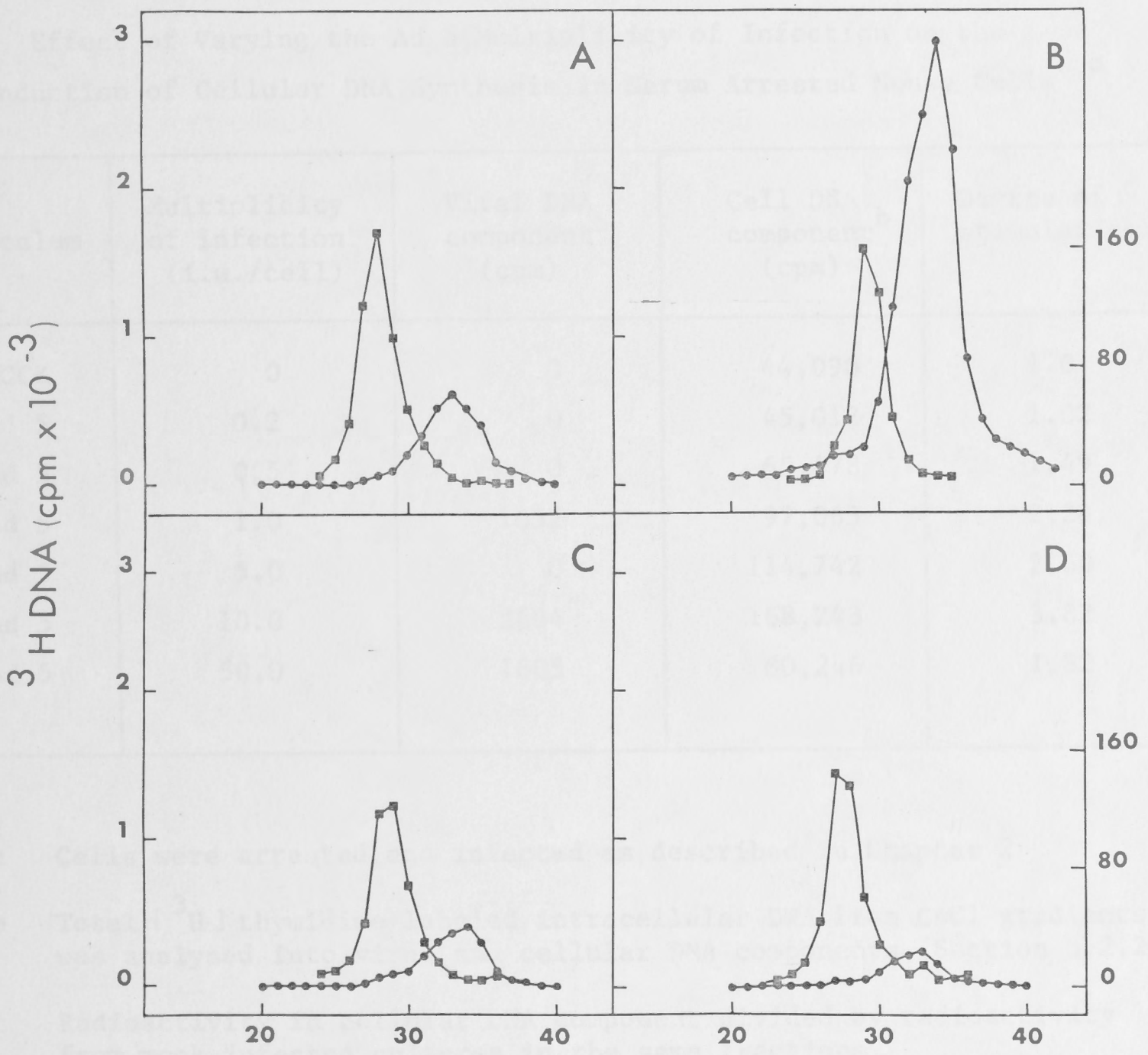

A
B
C
D
3
2
1
0
160
80
0
20
30
40
^{3}H DNA (cpm x 10^{-3})
^{32}P Ad 5 DNA (cpm)
FRACTION NUMBER

TABLE 3.3

Effect of Varying the Ad 5 Multiplicity of Infection on the Induction of Cellular DNA Synthesis in Serum Arrested Mouse Cells [a]

Inoculum	Multiplicity of infection (i.u./cell)	Viral DNA component[b] (cpm)	Cell DNA component[b] (cpm)	Degree of stimulation[c]
MOCK	0	0	44,090	1.0
Ad 5	0.2	0	45,012	1.02
Ad 5	0.5	0	65,478	1.49
Ad 5	1.0	1032	97,065	2.20
Ad 5	5.0	0	114,742	2.60
Ad 5	10.0	2684	168,243	3.82
Ad 5	50.0	1805	80,246	1.82

a Cells were arrested and infected as described in Chapter 2

b Total [^{3}H] thymidine labeled intracellular DNA from CsCl gradients was analysed into viral and cellular DNA components (Section 3.2.2)

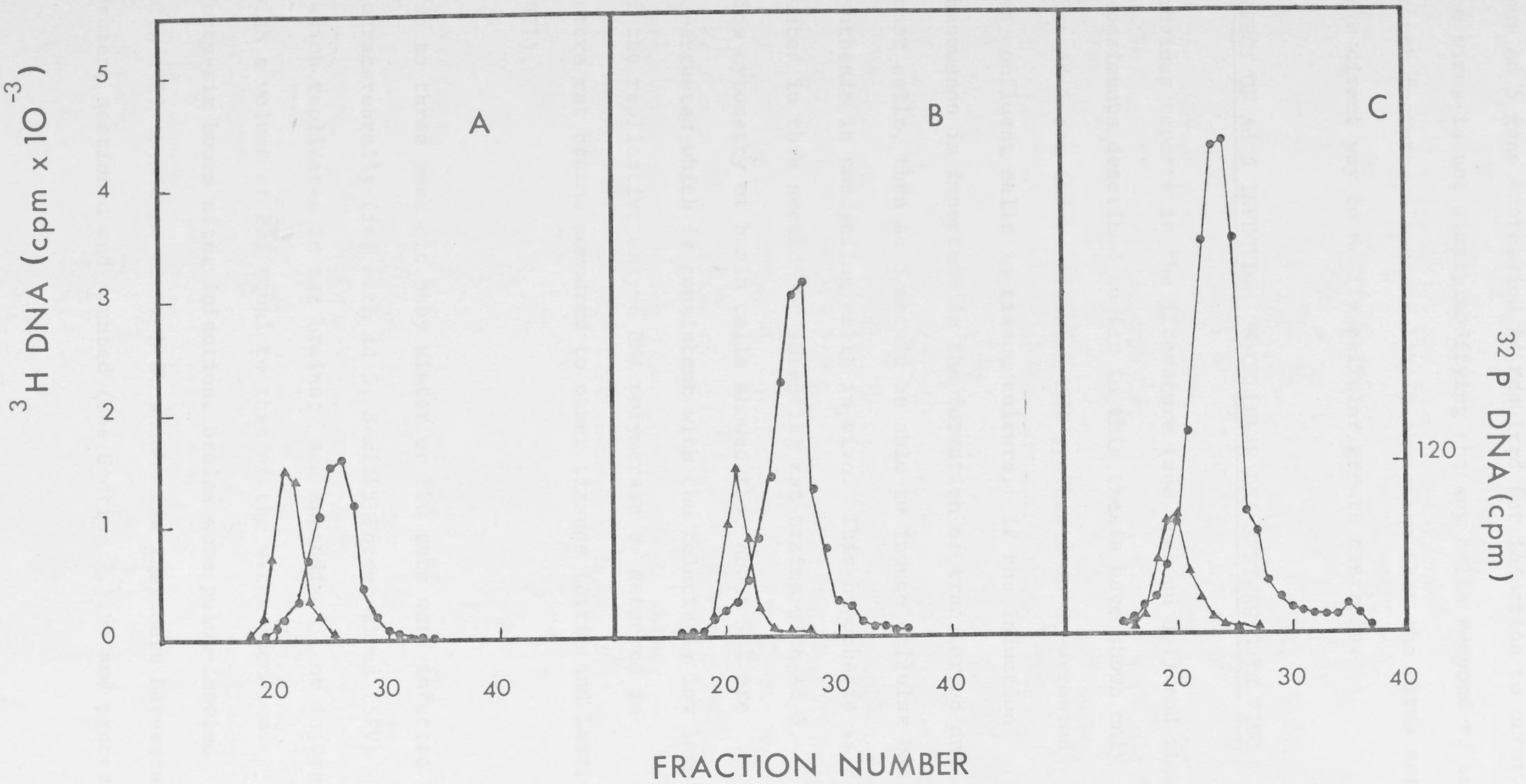
c Radioactivity in cellular DNA component divided by radioactivity from mock infected cultures in the same fractions

FIGURE 3.7

Induction of cellular DNA synthesis by Ad 5 in G_1-arrested rat cells in the complete absence of serum. Procedure was as outlined in the legend to Fig. 3.2, except that cells were labeled from 24 to 36 h after infection. (A) mock infected (0.2%); (B) Ad 5 infected cells in 0.2% serum medium; (C) Ad 5 infected cells without serum.

Symbols: ● [^{3}H] thymidine labeled DNA

▲ [^{32}P] labeled Ad 5 DNA marker


A
B
C
^{3}H DNA (cpm x 10^{-3})
32 P DNA (cpm)
0
1
2
3
4
5
120
20
30
40
FRACTION NUMBER

Thus Ad 5 gene expression is required for induction to occur and the virus is not simply modifying the way cells respond to serum growth factors. It seems likely therefore that the virus acts in a direct way to modify cellular growth controls.

3.3.3 EFFECT OF Ad 5 INFECTION ON CELLULAR DNA SYNTHESIS IN VIVO

Previous reports in the literature (see Section 3.1) and the experiments described so far in this thesis have shown only that Ad 5 can induce cellular DNA synthesis in G_1-arrested and confluent cells in tissue culture. If the induction phenomenon is important in the formation of transformed and tumor cells, then Ad 5 should be able to induce cellular DNA synthesis in noncycling cells in vivo. This hypothesis is tested in this section by infecting rat brains with Ad 5. Flow cytometry of brain cells showed that about 95% are G_1-arrested which is consistent with the relatively low levels of the replicative enzyme DNA polymerase α detected in mature rat brains compared to other tissues (Baril and Laszlo, 1971).

Two to three week old baby Wistar or PVG rats were infected intracerebrally (ic) with Ad 5, Semliki Forest Virus (SFV) (which replicates in rat brains; see Appendix I), or injected with a volume of PBS equal to that of the virus inoculum. Forty-six hours after infection, brains were pulse-labeled with [^{3}H] thymidine for 2 h at which time they were harvested, frozen, sectioned and mounted (see Section 2.1.5) and processed

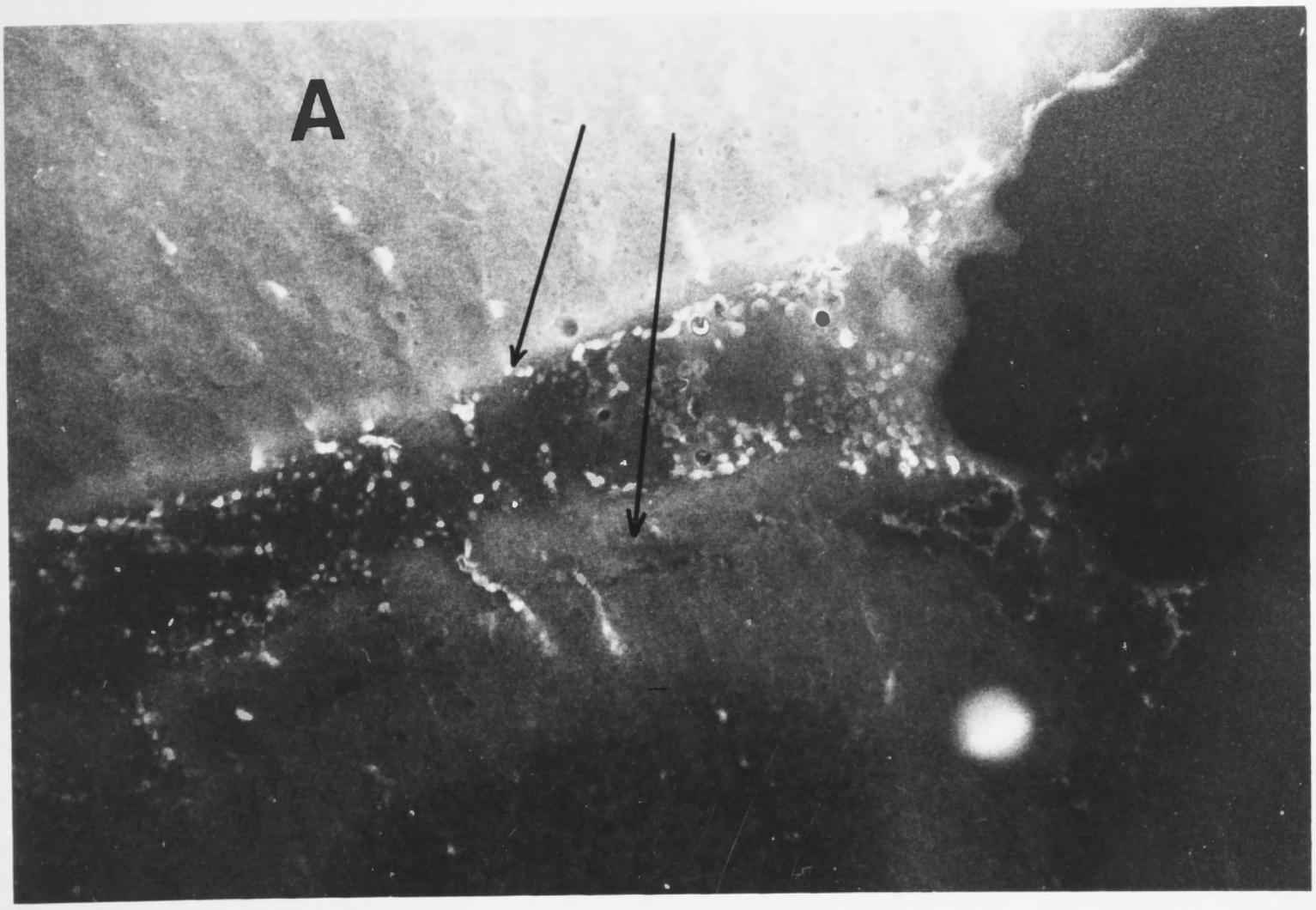
for autoradiography (see Section 3.2.4) or for measurement of Ad 5 early antigen expression (see Section 2.2.4).

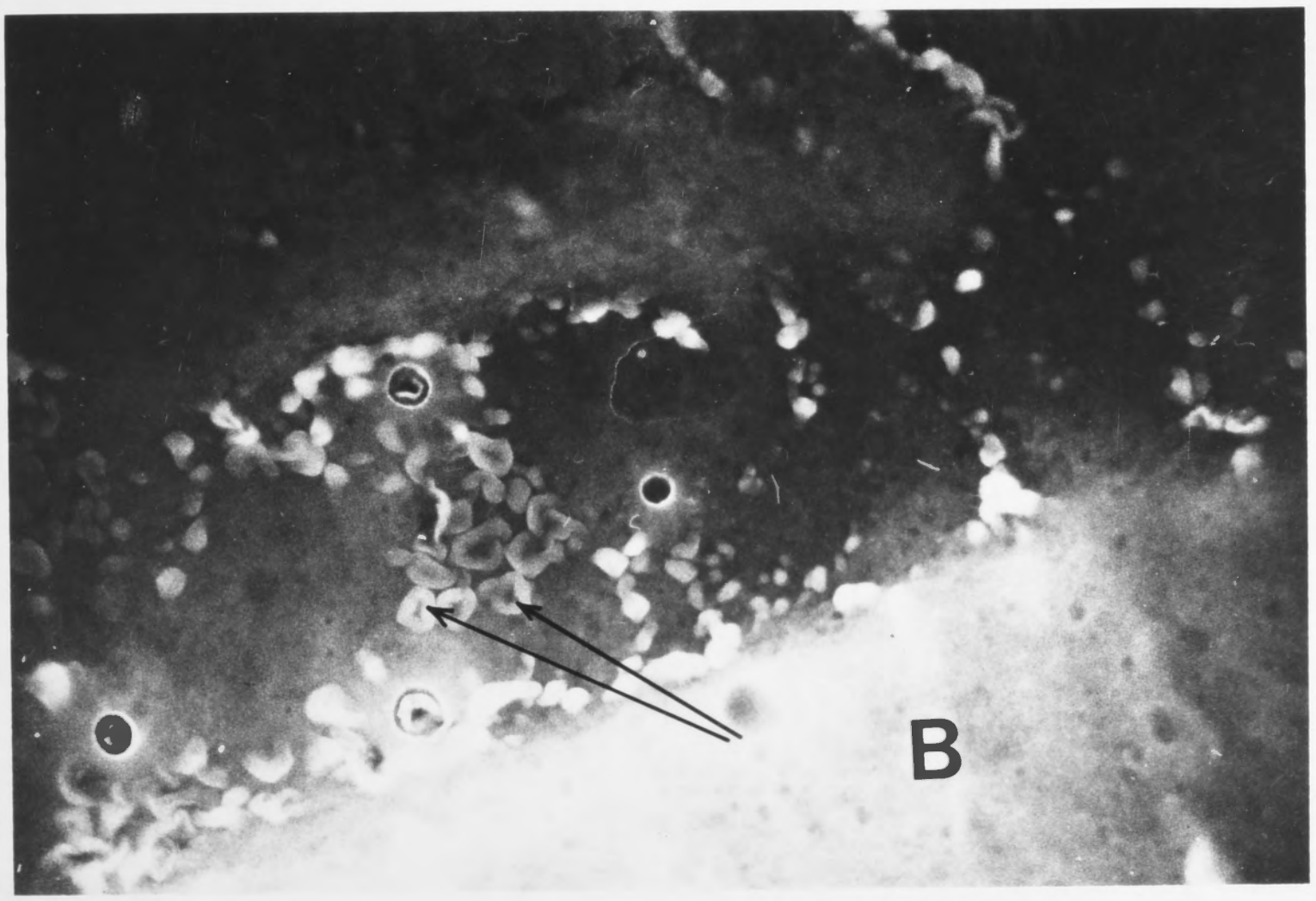
This experiment showed that Ad 5 caused a limited infection in many of the cells lining the ventricles (Fig. 3.8 A, B). This type of infection by Ad 5 has not been previously reported. In these same cells, autoradiography results showed that many cells were synthesising DNA (Fig. 3.8 C, D). This was not observed in brains inoculated with either PBS or SFV (Fig. 3.8 E, F). Thus it would appear that Ad 5 does stimulate cells to undergo DNA synthesis in vivo, although a small proportion of the nuclear labeling is likely to be due to replicating Ad 5 DNA as rat cells are semipermissive for virus replication (see Section 3.3.1). SFV infection does not induce this response although it does infect cells in the same region of the brain as well as neurones (Mims et al., 1973; I. D. Marshall, personal communication). Thus the induction of DNA synthesis by Ad 5 is probably not a nonspecific effect of virus infection.

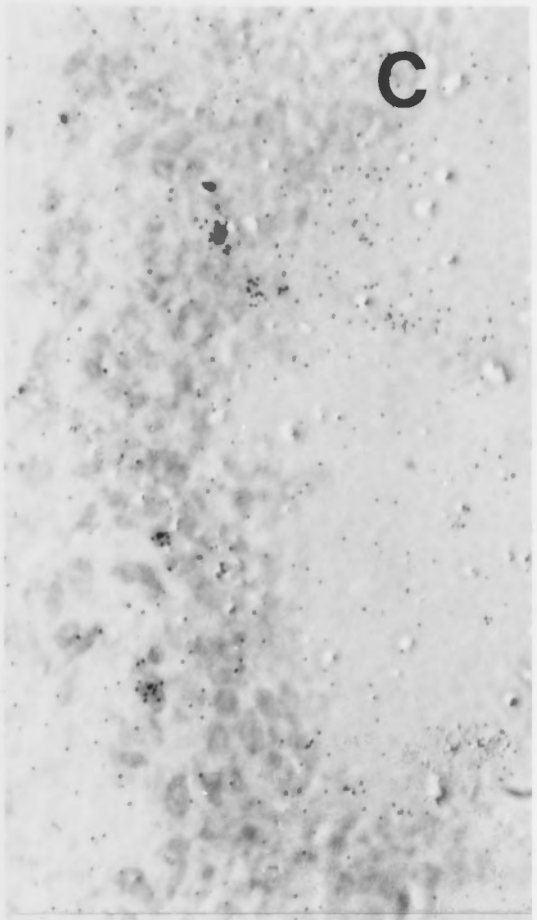
The cells stimulated into DNA synthesis by Ad 5 are most likely ependymal cells (which line the ventricles) and not neurones (M. L. Berger, personal communication). To be certain whether or not post-mitotic neurones are being induced into DNA synthesis by Ad 5 infection, neurones could be cultured and then infected with virus. I attempted this, but was unable to maintain neurones viable in culture for long enough to carry out the experiment.

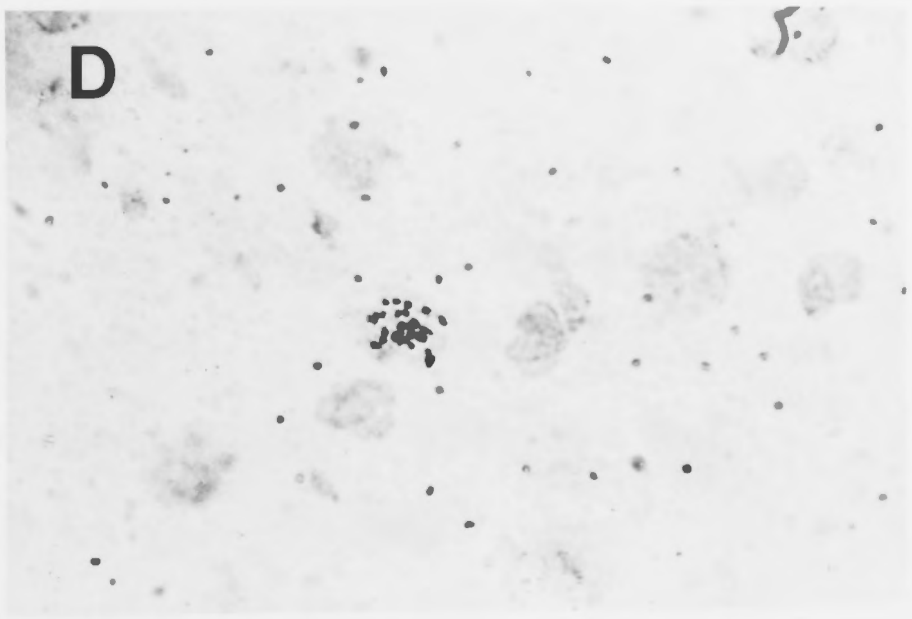
FIGURE 3.8

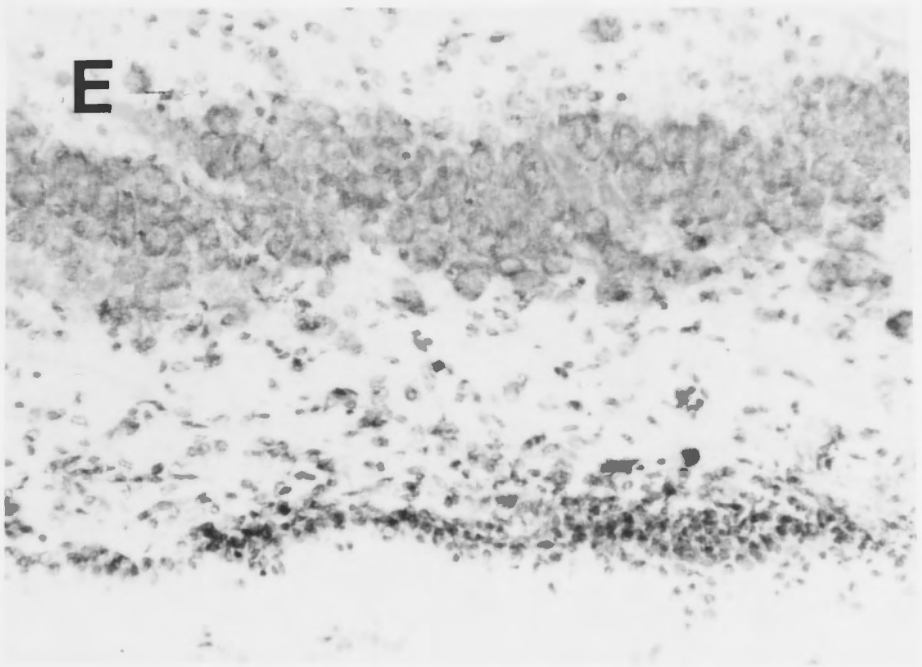
Infection and induction of DNA synthesis by Ad 5 in rat brain cells. Rat brains were infected with Ad 5, SFV, or mock infected with PBS, labeled with [^{3}H] thymidine, then harvested and fixed as described in the text. Brain sections were mounted on glass microscope slides then processed for viral antigen staining (using P antiserum) or for autoradiography. For details of procedure see Sections 2.1.5 (brain preparation), 2.2.4 (tests for viral antigens), and 3.2.4 (autoradiography). (A) Fluorescent P antiserum stained section from Ad 5 infected brain showing ventricular region of brain (arrows) and fluorescent cells lining the ventricles (ependymal cells); (B) As for (A), but taken at higher magnification. Arrows in this photograph refer to 2 cells showing positive fluorescence with P antiserum; (C) Ad 5 infected brain. Autoradiographic analysis showing nuclear labeling in some cells lining the ventricular region (325x); (D) One cell at 1300x magnification showing nuclear labeling; (E) PBS inoculated brain showing no autoradiographic labeling (325x); (F) SFV infected brain showing no autoradiographic labeling (325x).

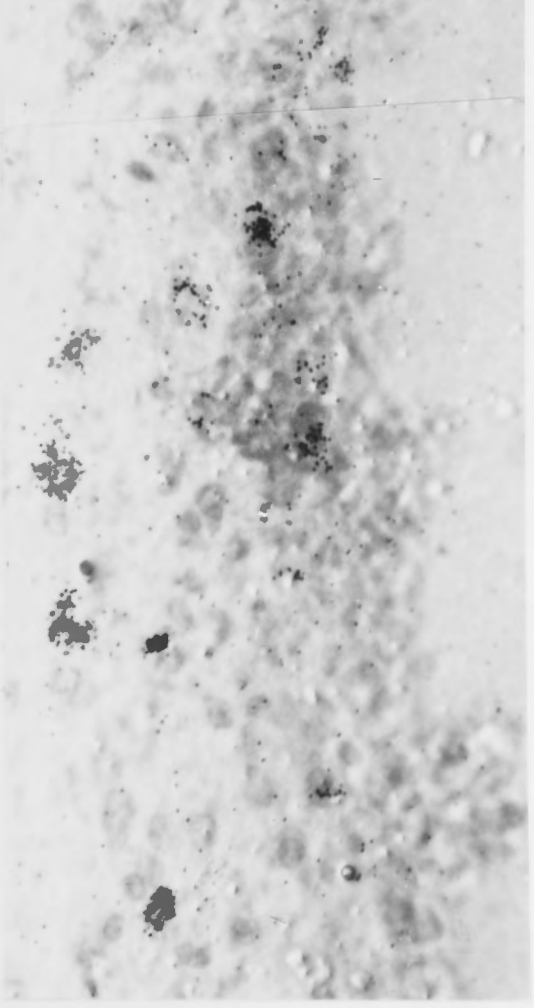

A

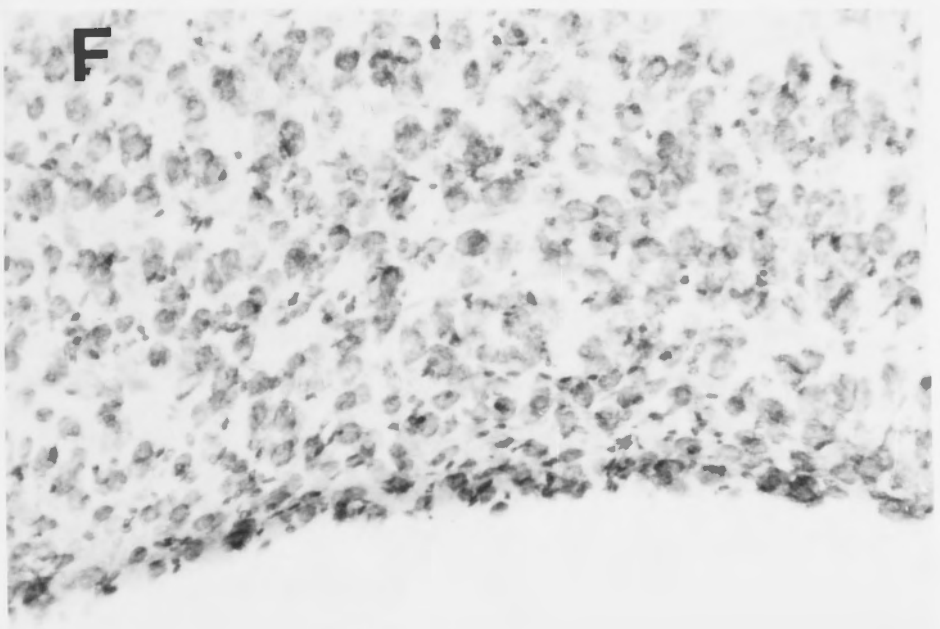

B


C


D


E




F

3.3.4 EFFECT OF ts MUTATIONS IN Ad 5 ON THE INDUCTION OF CELLULAR DNA SYNTHESIS

Results from previous sections in this chapter have provided strong evidence that Ad 5 induced cellular DNA replication requires the expression of at least one adenovirus gene product. In this section ts mutants of Ad 5 were used in order to investigate which specific region of the virus genome is responsible for inducing cellular DNA synthesis. The mutants studied were ts 36 and ts 37 which are defective for synthesis of the gene N product (early region 2B) and ts 125 which contains a mutation in the region coding for the DNA binding protein (early region 2A; see Section 3.1). These mutants have abnormal transformation properties at the nonpermissive temperature (Williams et al., 1974; Ginsberg et al., 1974; see Section 3.1).

All three ts mutants induced cellular DNA synthesis detectable by CsCl density gradient centrifugation of DNA from rat (Table 3.4) and mouse (Table 3.5) cells at the permissive (32.5^{o}C) temperature, at which viral DNA was also replicated. Cellular DNA replication was also induced at the nonpermissive temperature (39.5^{o}C) at which viral DNA replication was undetectable (rat cells) or reduced to less than 15% (mean 9.9%) of the wild-type Ad 5 control (mouse cells). Table 3.4 also illustrated that the induction of cellular DNA synthesis could be measured by autoradiography as an increase in the proportion of cells synthesising DNA (shown for Ad 5, ts 36, and ts 37 in rat cells). This measurement is only strictly valid at 39.5^{o}C

TABLE 3.4 Induction of cellular DNA synthesis by Ad 5, ts 36, ts 37, and ts 125 in G_1-arrested rat [a] cells

Inoculum	Time of labeling (h)	Temperature (^oC)	Viral DNA[b] Component (cpm)	Cell DNA[b] Component (cpm)	Degree of Stimulation[c]	% cells with radiolabeled nuclei	Degree of Stimulation by autoradiography[d]
MOCK	20-35	39.5	0	140,815	1.0	7	1.0
Ad 5	"	"	70,386	414,133	2.94	31	4.43
ts 125	"	"	0	469,424	3.33	ND	ND
ts 36	"	"	0	298,298	2.12	37	5.28
ts 37	"	"	0	670,827	4.76	23	3.29
MOCK	48-60	32.5	0	37,181	1.0	11	1.0
Ad 5	"	"	54,455	288,414	7.75	29	2.64
ts 125	"	"	32,701	250,675	7.67	ND	ND
ts 36	"	"	5,239	112,748	3.03	24	2.18
ts 37	"	"	33,539	305,355	8.16	23	2.09

a Cells were arrested and infected as described in Chapter 2

b Total [^{3}H] thymidine labeled intracellular DNA from CsCl gradients was analysed into viral and cellular DNA components (Section 3.2.2)

c Radioactivity in cellular DNA component divided by radioactivity from mock infected cultures

d Autoradiography results from a separate experiment

ND Not done

TABLE 3.5

Induction of cellular DNA synthesis by Ad 5, ts 36, ts 37, and ts 125 in G_1-arrested [a] mouse cells

Inoculum	Time of labeling (h)	Temperature (^{o}C)	Viral DNA[b] Component (c.p.m.)	Cell DNA[b] Component (c.p.m.)	Degree of Stimulation[c]
MOCK[d]	29-45	39.5	0	20,181	1.0
Ad 5[d]	"	"	3,640	117,219	5.81
MOCK	29-45	39.5	0	7,500	1.0
ts 125	"	"	512	34,729	4.63
ts 36	"	"	0	45,707	6.09
ts 37	"	"	566	44,260	5.90
MOCK[d]	24-48	32.5	0	45,568	1.0
Ad 5[d]	"	"	703	165,397	3.63
MOCK	24-48	32.5	0	9,214	1.0
ts 125	"	"	7,526	273,369	29.67
ts 36	"	"	753	152,720	16.57
ts 37	"	"	3,775	72,504	7.87

a, b, c As for Table 3.4

d Ad 5 induction tested in a separate experiment

when no viral DNA replication was occurring. At the permissive temperature for the ts mutants estimates from the equilibrium gradients (see Table 3.4) suggested that up to 16% of the observed DNA synthesis could be accounted for by viral DNA replication.

At 39.5^{o}C no or very little viral DNA synthesis was observed after infection by the ts mutants (see Tables 3.4 and 3.5) and late protein synthesis, which is dependent on viral DNA replication, was prevented. Since induction of cellular DNA synthesis was not reduced, it must be controlled by one or more early viral proteins. However, neither the gene N product nor the Ad 5 DNA binding protein can play an essential role in the induction of cellular DNA replication.

3.3.5 EFFECT OF DELETIONS IN Ad 5 ON THE INDUCTION OF CELLULAR DNA SYNTHESIS

The experiments described in the previous section with ts mutants of Ad 5 provided good evidence that the induction of cellular DNA replication is controlled by an early region gene product and excluded two of these regions as being responsible. This section examines the same question of which viral early region is responsible for inducing DNA replication by using two early region d1 mutants, d1 312 and d1 313 (see Section 3.1). They are deleted in early regions 1A and 1B respectively (Jones and Shenk, 1979a).

TABLE 3.6

Induction of cellular DNA Synthesis by

Ad 5, d1 312 and d1 313 in G_1-arrested mouse [a] cells

Inoculum	Time of labeling (h)	Viral DNA [b] component (cpm)	Cellular DNA [b] component (cpm)	Degree of stimulation [c]
MOCK	24-48	0	130,250	1.0
d1 312	"	0	120,214	NS
d1 313	"	2,425	257,569	1.98
Ad 5	48-72	164,983	494,950	3.80
d1 312	"	0	90,317	NS
d1 313	"	0	292,933	2.25

NS no stimulation (c.p.m. incorporation less than mock infected control)

a Cells were arrested and infected as described in Chapter 2

b Total [^{3}H] thymidine labeled intracellular DNA from CsCl gradients was analysed into viral and cellular DNA components (Section 3.2.2)

c Radioactivity in cellular DNA component divided by radioactivity from mock infected cultures

Equilibrium density gradient analysis of mouse cells infected with dl 312 and dl 313 demonstrated that dl 313, but not dl 312, stimulated arrested cells to synthesise cellular DNA (Table 3.6). Under the conditions of the experiment, replication of Ad 5 wild-type DNA was occurring and constituted 25% of the total [^{3}H] thymidine radioactivity. However, radioactivity from 48-72 h in mutant dl 313 infected cultures represented almost entirely cellular DNA (99% by 48 h; 100% by 72 h). Thus this mutant has a host-range defect in mouse cells for viral DNA replication but not for the induction of cellular DNA replication. This result therefore suggests that early region 1B is not responsible for inducing cellular DNA synthesis, but is required for viral DNA replication.

The negative result observed with dl 312 suggests a priori that early region 1A codes for some gene product which is responsible for inducing cellular DNA replication. However, results from Berk et al.,(1979) and Jones and Shenk (1979b) showing that early region 1A regulates expression of all the other adenovirus early regions, suggests that an alternative explanation of the dl 312 result is possible. These results are discussed in detail in Section 3.4.

3.3.6 DNA SYNTHESIS IN SERUM STIMULATED AND Ad 5 INFECTED RODENT CELLS AFTER CYCLOHEXIMIDE TREATMENT

Cycloheximide is an inhibitor of protein synthesis which has been usefully employed by many groups to investigate the protein synthesis requirements for DNA synthesis. Most eukaryotic cells

with the possible exception of yeasts (Hereford and Hartwell, 1973), require protein synthesis both to begin and to continue DNA replication (reviewed in Prescott, 1976). On the other hand, whilst protein synthesis is required to begin adenovirus DNA replication, it is not required for its continuation (Horwitz et al., 1973). Thus all the cellular and viral proteins (early region proteins) required in the Ad 5 DNA replication complex, once synthesised are sufficient to continue synthesis of the adenovirus replicon.

In this section I have examined the protein synthesis requirements for initiation and continuation of cellular DNA replication, in cells in which DNA replication has been induced either by serum treatment or infection with Ad 5.

G_1-arrested mouse and rat cells were infected with Ad 5 or serum treated, and 1.0 µg/ml cycloheximide was then added. All cultures were subsequently labeled with [^{3}H] thymidine and the DNA analysed by density gradient centrifugation. Results from these experiments (Table 3.7) showed that Ad 5 induced and serum induced DNA replication are inhibited by cycloheximide added immediately after stimulation. These results imply that protein synthesis is necessary for induction of cellular DNA replication by Ad 5 and by serum. If DNA synthesis was allowed to begin and cycloheximide (1.0 µg/ml) was then added to cultures at 36 h after infection or serum addition, both serum induced and Ad 5 induced cellular DNA synthesis were still inhibited (Table 3.7). Under these conditions adenovirus DNA synthesis continued normally

TABLE 3.7 Effect of Cycloheximide on Cellular and Viral DNA Synthesis induced by Ad 5 or serum in G_1-arrested [a] rat and mouse cells

Cell Type	Inoculum	Addition of cycloheximide	Time of cycloheximide (h)	Viral DNA[b] Component (c.p.m.)	Cell DNA[c] Component (c.p.m.)	Degree of Stimulation
Rat	Mock	-	-	0	4,643	1.0
	Ad 5	-	-	50,886	102,197	22.01
	Ad 5	+	0	1,081	500	NS
	Ad 5	+	36	50,372	10,595	2.28
	10% FCS	-	-	0	143,390	30.88
	10% FCS	+	36	0	5,796	1.25
Mouse	Mock	-	-	0	6,710	1.0
	Ad 5	-	-	5,458	23,782	3.54
	Ad 5	+	0	1,600	199	NS
	Ad 5	+	36	5,099	3,467	NS
	10% FCS	-	-	0	44,464	6.63
	10% FCS	+	0	0	1,500	NS
	10% FCS	+	36	0	3,726	NS

a, b, c As for Table 3.6
After infection or serum stimulation cultures were treated with 1.0 µg/ml cycloheximide at indicated times. They were labeled with [^{3}H] thymidine from 36 to 48 h later and then DNA was extracted and analysed by gradient centrifugation

NS no stimulation (c.p.m. incorporated less than mock infected control)

(Table 3.7) as has been reported previously (Horwitz et al., 1973). Thus, continuing cellular DNA synthesis requires continued protein synthesis after induction by serum or by virus, but viral DNA synthesis becomes independent of protein synthesis once begun. Cycloheximide may inhibit synthesis of a cellular protein which is continuously required for cellular but not for viral DNA replication, irrespective of the agent initiating cellular DNA replication.

3.3.7 CELL CYCLE ANALYSIS OF G_1-ARRESTED RAT CELLS STIMULATED WITH SERUM OR Ad 5 INFECTION

Results of experiments presented in previous sections have shown that Ad 5 and some mutants can overcome the low serum arrest point (Pardee, 1974) and induce cells into the DNA synthesis phase (S phase) of the cell cycle. Such results have shown only that DNA synthesis is initiated, but no experiment provides evidence as to whether a complete round of chromosome replication occurs. In Ad 12 infected hamster cells, only a single round of cellular DNA replication was observed to occur (Strohl, 1973). However a second round was induced in SV40 infected chinese hamster cells (Hirai et al., 1971) within a single cell cycle. In this section I have used the techniques of microspectrophotometry and flow cytometry to answer this question for the induction of cellular DNA synthesis by Ad 5 in G_1-arrested rodent cells. The details of these methods are discussed in Section 3.2.5 and in the following chapter (flow cytometry).

TABLE 3.8

Cell cycle stages of Ad 5 infected and serum treated G_1-arrested [a] rat cells determined by autoradiography [b] and microspectrophotometry [c]

Inoculum	P-antiserum positive cells (%)	Approximate % of cells in each stage of the cell cycle 55 h after serum treatment or infection			
		G_1	S	G_2 + M	$>G_2$ diploid DNA content
Mock	0	ND[d]	4.7[e]	ND	ND
Serum	0	46.4	28.5	25.1	0
Ad 5	40	27.0	35.6	31.0	6.4

a Rat cells were arrested and infected as described in Chapter 2

b A total of 1000 cells were scored for the presence of radiolabeled nuclei to estimate the proportion of cells in S phase

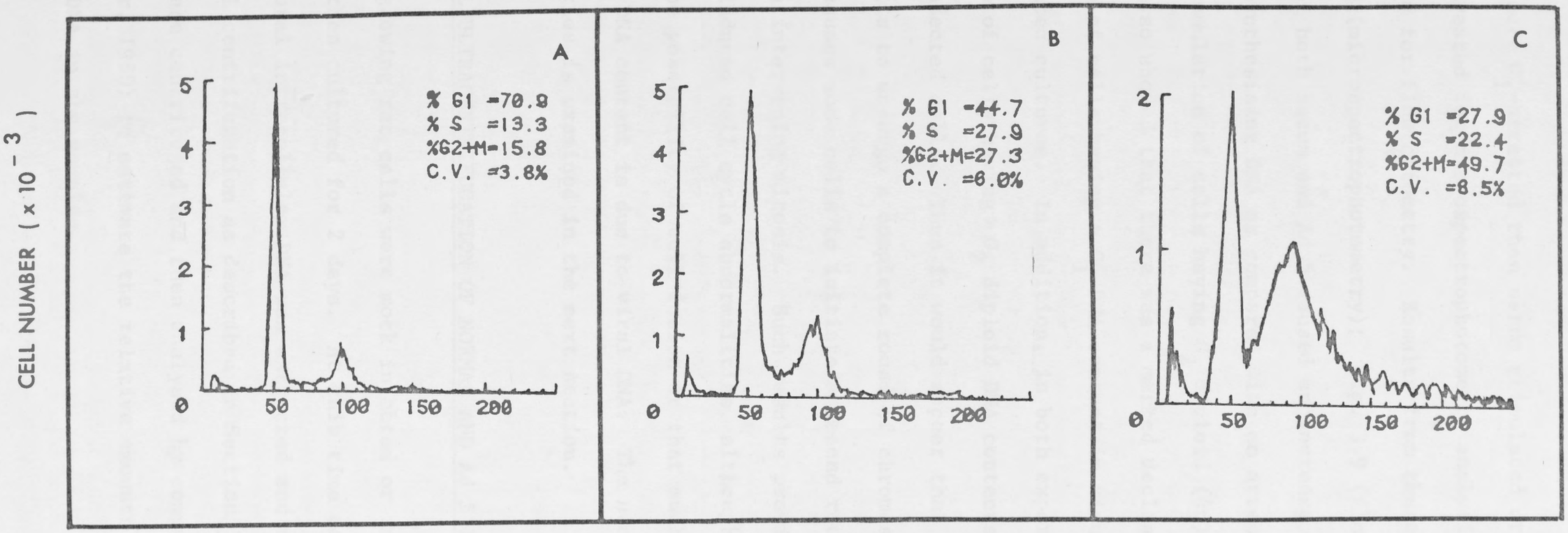
c 50 cells without nuclear grains were analysed by microspectrophotometry to determine the relative proportion of cells with G_1, G_2 + M, or $> G_2$ diploid DNA contents (see Section 3.2.5)

d ND = not done

e Based on autoradiography data only

FIGURE 3.9

DNA contents of Ad 5 infected and serum treated rat cells determined by flow cytometry. Rat cells were arrested in G_1 and infected or serum treated as described in Chapter 2. After 48 h cells were harvested and stained, and their DNA contents were analysed by flow cytometry (see Chapter 4). (A) Mock infected; (B) Serum treated; (C) Ad 5 infected (30 to 35% P antiserum positive at this time). "Channel Number" indicates the relative DNA content of cells, and the peak in channel 18 represents a chicken erythrocyte DNA marker. C.V., coefficient of variation of the G_1 peak. (The proportions of cells in different stages of the cell cycle were calculated with a program which does not include cells with DNA contents $>G_2$ diploid (4n) (Milthorpe, 1980)).


A
% G1 =70.9
% S =13.3
%G2+M=15.8
C.V. =3.8%
B
% G1 =44.7
% S =27.9
%G2+M=27.3
C.V. =6.0%
C
% G1 =27.9
% S =22.4
%G2+M=49.7
C.V. =8.5%
CELL NUMBER (x10⁻³)
CHANNEL NUMBER

Rat cells were G_1-arrested then serum stimulated or infected with Ad 5 and treated for microspectrophotometry analysis or harvested and stained for flow cytometry. Results from these two experiments (Table 3.8 (microspectrophotometry); Fig. 3.9 (flow cytometry)) showed that both serum and Ad 5 caused an increase in the proportion of cells synthesising DNA as compared with an arrested control and an accumulation of cells having G_2 diploid (4n) DNA contents. Fig. 3.9 also showed that there was a marked decline in the proportion of cells having a G_1 DNA content in virus infected and serum treated cultures. In addition, in both experiments a proportion of cells having $> G_2$ diploid DNA contents was observed in Ad 5 infected cells. Thus it would appear that Ad 5 not only causes cells to undergo a complete round of chromosome replication but also causes some cells to initiate a second round of replication without an intervening mitosis. Such results provide evidence for Ad 5 induced cell cycle abnormalities, although they do not exclude the possibility of cell fusion or that much of the increased DNA content is due to viral DNA. The second of these possibilities is examined in the next section.

3.3.8 ANALYTICAL ULTRACENTRIFUGATION OF NORMAL AND Ad 5 INFECTED RAT CELLS

Randomly growing rat cells were mock infected or infected with Ad 5 and then cultured for 2 days. At this time cells were lysed and the total intracellular DNA was extracted and treated for analytical centrifugation as described in Section 3.2.3. DNA samples were centrifuged and then analysed by computer (see method of Reisner, 1980) to estimate the relative amounts of viral and cellular DNA in the samples.

Calculations based on previously published work * suggested that at best only 1-2% of the total intracellular DNA could be accounted for by Ad 5 DNA copies; however results from this experiment (Fig. 3.10) demonstrated Ad 5 DNA constituted 11% of the total. In addition, repeat experiments showed that the amount of Ad 5 DNA in rat cells ranged from 5-18% of the total DNA present. Assuming that the G_2 diploid DNA content is represented by 100 arbitrary fluorescence units (as measured by channel number) (Fig. 3.9) then Ad 5 DNA could only account for an increase in fluorescence ($>G_2$ diploid) of less than 20 channels. Yet increases are observed of >100 fluorescent channels suggesting that most of the observed abnormal DNA contents is due to cellular DNA. This conclusion is confirmed in the next chapter with experiments in which DNA content analyses and analytical ultracentrifugation of ts mutant infected cells is done.

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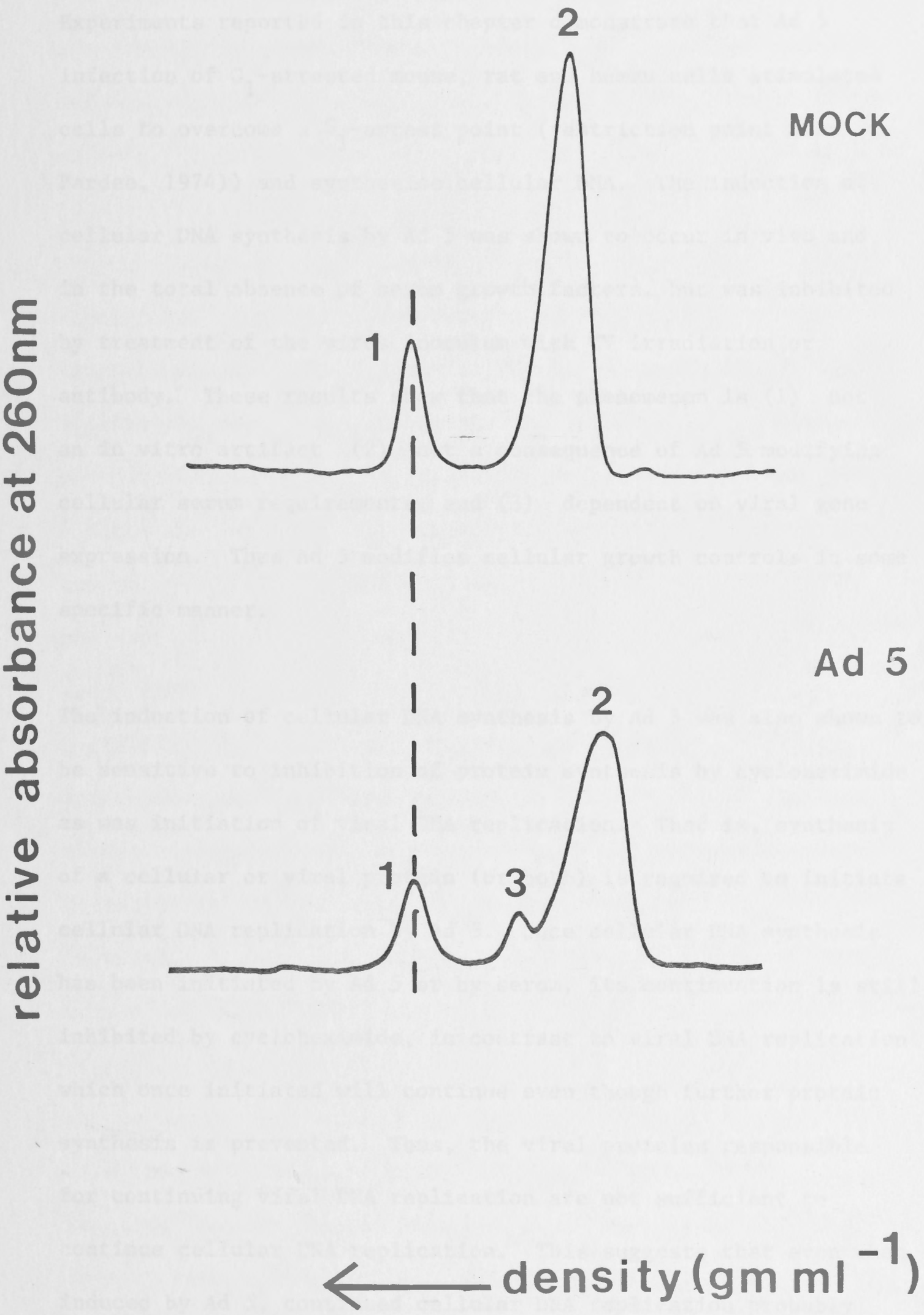
* See Appendix II

FIGURE 3.10

Analytical centrifugation of DNA extracted from Ad 5 infected rat cells. Approximately 10^6 rat cells were seeded into dishes and 24 h later they were infected with Ad 5 (10 iu/cell) or mock infected. 48 h after infection DNA was extracted (see Sections 3.2.2. and 3.2.3.) and 2-3 µg plus Micrococcus lysodeikticus DNA marker, was centrifuged in a Beckman Model E ultracentrifuge. (A) mock infected cells plus DNA marker; (B) Ad 5 infected cells plus DNA marker.

Labels:
1 Micrococcus lysodeikticus DNA (p = 1.731)
2 Rat cell DNA (p = 1.7046)*
3 Ad 5 DNA (p = 1.7142)*

* These estimated DNA densities are in quite close agreement with published values: 1.714 for Ad 5 DNA (Piña and Green, 1965), and 1.701 for mouse cell DNA (Corneo et al., 1966).


relative absorbance at 260nm
MOCK
1
2
Ad 5
1
3
2
density (gm ml^{-1})

3.4 DISCUSSION AND CONCLUSIONS

Experiments reported in this chapter demonstrate that Ad 5 infection of G_1-arrested mouse, rat and human cells stimulates cells to overcome a G_1-arrest point (restriction point (see Pardee, 1974)) and synthesise cellular DNA. The induction of cellular DNA synthesis by Ad 5 was shown to occur in vivo and in the total absence of serum growth factors, but was inhibited by treatment of the virus inoculum with UV irradiation or antibody. These results show that the phenomenon is (1) not an in vitro artifact (2) not a consequence of Ad 5 modifying cellular serum requirements, and (3) dependent on viral gene expression. Thus Ad 5 modifies cellular growth controls in some specific manner.

The induction of cellular DNA synthesis by Ad 5 was also shown to be sensitive to inhibition of protein synthesis by cycloheximide as was initiation of viral DNA replication. That is, synthesis of a cellular or viral protein (or both) is required to initiate cellular DNA replication by Ad 5. Once cellular DNA synthesis has been initiated by Ad 5 or by serum, its continuation is still inhibited by cycloheximide, in contrast to viral DNA replication which once initiated will continue even though further protein synthesis is prevented. Thus, the viral proteins responsible for continuing viral DNA replication are not sufficient to continue cellular DNA replication. This suggests that even when induced by Ad 5, continued cellular DNA replication probably requires continued synthesis of a cellular protein.

The observations that the mutants ts 36, ts 37, ts 125, and d1 313 are not defective for the induction of cellular DNA synthesis, under conditions when they are defective for viral DNA replication, argue against the gene N product, the DNA-binding protein, and early region 1B products being responsible for inducing cellular DNA synthesis. The transcriptional unit(s) responsible for this phenomenon could therefore be located in one of the other early regions 1A, 3 or 4, or it could be one of the other gene products mapping in early region 2B (Lewis and Matthews, 1980; Stillman et al., 1981). The negative result obtained with d1 312, which is deleted in early region 1A, still does not provide a clear answer as to which transcriptional unit(s) control the induction of cellular DNA synthesis, as this region regulates the expression of all other early regions (Berk et al., 1979; Jones and Shenk, 1979b). Thus, this result again allows the possibility that early regions 1A, 3 or 4 are responsible for inducing cellular DNA synthesis. Using the Ad 2 deletion mutant Ad 2 Δp 305, which is deleted in part of early region 3, Rossini et al.,(1981) showed that this region of the Ad 2 genome is nonessential for induction of cellular DNA synthesis in G_1-arrested hamster cells. This result argues against early region 3 being responsible for inducing cellular DNA synthesis. Rossini and co-workers (Rossini et al., 1981) and Shiroki et al.,(1981) also obtained the same results for the ts and d1 mutants as reported in this chapter with the exception of ts 125, which Rossini et al., (1981) reported to be negative for induction of hamster cell DNA synthesis at the restrictive temperature.

My results do however demonstrate a clear defect in viral DNA replication at the nonpermissive temperature in ts 125 infected rat and mouse cells. Such cells do not stain with P antiserum, which reacts primarily with the DNA binding protein, although they do stain positively if ts 125 infected cells are incubated at the permissive temperature (see Appendix III). Both viral DNA and cellular DNA replication are also observed at the permissive temperature (Tables 3.4 and 3.5). This result is confirmed by flow cytometry (see Chapter 4). One possible explanation of the difference between the results reported by Rossini et al., (1981) and those reported in this chapter might be due to a difference in the cells used, as some cellular protein(s) do appear to be necessary for adenovirus to induce cellular DNA replication (see Section 3.3.6). The results from these two groups (Rossini et al., 1981; Shiroki et al., 1981) suggest overall that early region 1A is responsible for the induction of cellular DNA synthesis, as do the results in this chapter, but still do not exclude early region 4. Results presented in the next chapter provide additional evidence that early region 1A is the critical region responsible for the induction of cellular DNA synthesis.

Microinjection experiments with liposomes (Allebach et al., 1980), in which a mixture of several early proteins precipitated with anti-Ad 12 T antiserum was injected into quiescent cells, showed induction of cellular DNA synthesis. Two of the "T" antigens (58K T, 15K t) have been assigned to mRNA molecules encoded by early region 1B (Harter and Lewis, 1978; Lewis et al., 1979; Spector et al., 1979). However, since Allebach's experiments were not carried out with specifically purified T or t antigens,

no firm conclusions can be drawn about the gene product(s) responsible for inducing cellular DNA synthesis. Nevertheless, the results do suggest early region 1 (1A and/or 1B) involvement. By analogy with the SV40 gene A, which is involved in transformation and also encodes the SV40 T antigen, that has clearly been shown to stimulate cellular DNA replication (Graessmann et al., 1980), it might also be expected that the adenovirus transforming region would be responsible for inducing cellular DNA replication, i.e. early regions 1A and/or 1B.

On the basis of these results alone, however, whilst it does seem likely that early region 1 (and probably 1A) is the region of the genome responsible for inducing cellular DNA synthesis, until other mutants are available, particularly in early regions 2B and 4, it is not possible to make any more definite conclusions. One useful approach to resolve this confusion, would be to generate specific deletions in these two regions by cutting the DNA with an appropriate restriction endonuclease and using controlled digestion of the cut ends with BAL31-111 nuclease (Legerski et al., 1978). Mutants could then be isolated using the techniques described by Jones and Shenk (1978).

Early region 1 (1A and 1B) is the only region of the Ad 5 genome that is mandatory for transformation, as it can be established with a restriction endonuclease fragment of Ad 5 DNA containing only this region (Graham et al., 1977). In addition, deletion mutants in these 2 regions (d1 312 and d1 313) are both defective for transformation of rodent cells (Jones and Shenk, 1979a).

Since it seems likely that the induction of cellular DNA synthesis is controlled by early region 1A the initiation of transformation may require cells to be induced into a cell cycle. However, this is clearly not the only requirement as ts 36, ts 37 (Williams et al., 1974), and ts 125 (Ginsberg et al., 1974), whilst not being defective for transformation under restrictive conditions, all have altered transformation frequencies compared to wild-type Ad 5, but do not affect cellular DNA synthesis (this is discussed in more detail in Section 3.1). Thus cellular DNA replication might be essential for transformation, but these other regions (2A and 2B) must affect transformation frequency through some other mechanism.

Once DNA synthesis has been initiated by Ad 5 infection, results from Table 3.8 and Fig 3.9 would seem to show that cells undergo a complete round of chromosome replication and in some cells (involving up to a third of the total population of cells in culture) a second round without an intervening mitosis.

Model E analysis (Fig. 3.10) of DNA from Ad 5 infected cells suggests that replicating Ad 5 DNA is able to account for $< 20\%$ of the total increase in observed DNA content . Thus, most of the observed increase is likely to be due to cellular DNA. This can be explained by reinitiation of DNA synthesis as suggested above, or by adenovirus induced cell fusion. There are no reports in the literature of adenovirus causing fusion, and experiments in this laboratory carried out under the conditions described in this thesis, failed to detect any fusion occurring

in greater frequency than in uninfected controls (J.D. Murray, personal communication). Thus a reasonable conclusion is that Ad 5 causes cellular DNA replication to be reinitiated in many cells without completion of chromosome segregation and cytokinesis. This adenovirus induced polyploidy is investigated in detail in the next chapter.

CHAPTER 4

DNA CONTENT ANALYSIS OF ADENOVIRUS INFECTED RODENT CELLS

4.1 INTRODUCTION

Transformation of animal cells in culture is a general term which often includes the processes whereby cells are able to grow under conditions which severely restrict normal cells, such as serum or calcium deprivation, crowding or conditions which prevent cell anchorage. Such selection may be important in vivo in tumorogenesis as the majority of cells in most tissues are held in a nonproliferative (G_1 or G_o) stage of the cell cycle. It might be expected therefore that genetic alterations would occur during transformation including altered gene expression as well as content and structural changes. All such genetic alterations have been reported for some virus transformed and virus induced tumor cells (see reviews by Wolman and Horlound, 1975; Rapp and Westmoreland, 1976). In addition, many viruses, including members of the papova-, adeno-, and herpes groups cause severe chromosome damage during lytic infections (Stich and Yohn, 1970) and in some cases during nonlytic infections (see McDougall et al., 1974). Much of the damage appears to be random but in some cases specific chromosome lesions have been shown to occur. To cite one example, Ad 12 causes a single break on chromosome 17 during a permissive infection of human cells (zur Hausen, 1967).

Aside from random or nonrandom chromosome alterations, Ad 12 has been shown to increase the proportion of polyploid metaphases in human cells, which by 24 h after infection, is as high as 22% of the total metaphase population examined

(McDougall et al., 1974). Such numerically altered karyotypes appear to be a common feature associated with oncogenic virus infection of cells, and several examples of hyperploidy are cited by Bartsch (1970) for SV40, polyoma, and RSV. Hyperploidy can be explained both by a gain in chromosome number and by endoreduplication (MacKinnon et al., 1966). Alterations to cellular DNA content by virus infection have probably been best studied for SV40. These are discussed in detail below.

4.1.1 SV40, ABERRANT CELL GROWTH, and TRANSFORMATION

Early experiments from Defendi's group (Hirai et al., 1971) using 5-bromodeoxyuridine labeling of replicating DNA showed that two rounds of DNA synthesis occurred within one cell cycle in SV40 infected chinese hamster cells. In addition, using time-lapse cinemicrography of single cells they confirmed their observations that SV40 causes the reinitiation of cellular DNA synthesis without an intervening mitosis (Lehman and Defendi, 1970). Subsequently, these experiments were repeated with large numbers of cells using the technique of flow cytometry (Horan et al., 1974) and in diploid mouse peritoneal macrophages using cytophotometry (Lehman et al., 1971). Thus this effect of SV40 is not confined solely to a single cell type.

These increases in cellular DNA content do not necessarily imply an increase in chromosome number (see above). In fact, they only imply an increase in the number of binding sites for DNA-specific fluorochromes which is taken to mean an increase

in DNA. Thus it is also important to show increases in cellular ploidy by SV40 infection. This was shown by Lehman (1974) for chinese hamster cells and diploid mouse embryo fibroblasts. In infected chinese hamster cells 5-6% of polyploid metaphases were observed which was consistently 3-6 times greater than for controls. A large proportion (24-28%) of polyploid metaphases exhibited chromosomal abnormalities including complete breaks, dicentrics, and acentric fragments.

The relationship between polyploidy and transformation in SV40 infected and SV40 transformed chinese hamster cells was also examined by this group (Hirai et al., 1974). This was investigated in two ways: (1) DNA content analyses of SV40 transformed cells were carried out, and (2) the transformation ability of fractions of SV40 infected cells was tested, after separation by size on a bovine serum albumen gradient. Some of these fractions were enriched for polyploids. Results from these experiments showed that the majority of SV40 transformed cells exhibited polyploid DNA contents, and fractions of SV40 infected cells enriched in polyploids showed higher transforming frequencies. Such results suggest that the formation of polyploid containing cells may be an important early event in the initiation of transformation by SV40 and possibly by other oncogenic DNA viruses. Results from the above experiments (in particular Hirai et al., 1974) and more recently from others (Hiscott and Defendi, 1979), using flow cytometry of cells infected with wild-type SV40 and a ts A mutant (ts A 58), showed a correlation between SV40 induced cell

cycling (and polyploid formation) and expression of the gene A product (large T antigen). This gene product is responsible for inducing cellular DNA synthesis (Graessmann et al., 1980; Tijian et al., 1978; Hiscott and Defendi, 1979; and other studies cited in Section 3.1). Some ts A 58 transformed mouse cells (e.g. the A 21 cell line) were also observed to lose their transformed phenotype at the nonpermissive temperature for expression of the gene A product (Hiscott and Defendi, 1979).

The relationship between an SV40 stimulated cell cycle and polyploidy, gene A expression, and transformation is not however as clear as is indicated by the experiments discussed above. Mutants of SV40 deleted in the region between 0.54 and 0.59 on the genome, which are positive for large T antigen expression, but defective for small t antigen expression have been observed to have altered transforming abilities in rat cells (Sleigh et al., 1978). The extent of transformation in these experiments was shown to vary depending upon the assay used to measure transformation, but in general the ability of the mutants to transform cells fell as the size of the deletion increased. Thus in these experiments, the mitogenic ability of SV40 was separated from its transforming ability.

Three classes of SV40 transformants have been isolated by Seif and Martin (1979) after infection of rat fibroblasts with another ts gene A region mutant of SV40 (ts A 209). Some mutants were temperature sensitive for the transformed phenotype and others were temperature insensitive. Foci appearing

after infection of rapidly growing cells were temperature sensitive whereas foci isolated from infection of confluent rat cells were temperature insensitive. Transformants isolated by the semi-solid agar assay were all temperature insensitive. That is, the state of the cell in the first few days after infection plays an important role in determining the type of SV40 induced transformant isolated. Thus some SV40 transformants would appear to be A gene dependent and others independent. In primary and established SV40 transformed mouse cells Bender and Brockman (1981) showed differences in the arrangement of integrated SV40 DNA sequences. This illustrates a flexibility in the way viral sequences integrate into the cell chromosome and presumably reflect differences in viral gene expression in transformed cells. This type of explanation might account for the different phenotypes of transformed cells isolated by Seif and Martin (1979).

In addition to transformed cells isolated by infection with ts A mutants,Seif and Martin (1979) also isolated some transformed foci using mutants deleted in the small t antigen coding region. This suggests in contrast to the results of Sleigh et al., (1978), that t antigen is not absolutely critical for establishing transformation.

Recent collaborative experiments from a number of laboratories, which were reported in the 1979 Cold Spring Harbor Symposium (see Martin et al., 1979a) also cast doubt on the generality with which the SV40 small t antigen was involved in transformation.

They infected chinese hamster lung cells and 3T3 mouse cells with SV40 wild-type and mutants with altered or defective small t protein expression and "looked" for the appearance of transformed cells, without using the strongly selective procedures of low calcium or agar growth media. Using this procedure they isolated a spectrum of different transformed cells from both wild-type and mutant infected cultures which varied considerably in their growth properties. These studies suggested that small t antigen is not crucial for cellular transformation to occur, and once again emphasised the importance of the selection criteria used in the isolation of transformants. In other experiments, hamster cell lines which were transformed by mutants that were totally defective for small t antigen synthesis were shown to be tumorogenic in nude mice (Martin et al., 1979b), and when 10^7 pfu/ml of these virus mutants were inoculated into newborn hamsters they formed tumors in as many as 70-90% of the animals by 1 year after infection (Lewis and Martin, 1979). Martin et al (1979b) have suggested that small t antigen may act as a growth-factor substitute based on the observations that mutants defective for t antigen synthesis could transform growing but not quiescent hamster cells. Under growth inhibitory conditions such t defective mutants will transform cells if supplied with platelet-derived growth-factor. These observations suggest that under certain conditions small t antigen may be required for the maintenance of transformation which depends very much on the growth conditions of transformed cells in tissue culture.

From these experiments it appears that the genetic control of SV40 transformation is extremely complex and it is therefore hard to form any definitive conclusions. It does appear, however, that more than one viral gene product is involved and the physiological state of the cell is important; and at least for some transformants, cellular mitogenesis may be a necessary requirement for transformation.

4.1.2 ADENOVIRUS, TRANSFORMATION, AND MITOGENESIS

Results presented in the previous chapter using Ad 5 ts and d1 mutants, and others discussed in that chapter (Minekawa et al., 1976; Rossini et al., 1981; Shiroki et al., 1981) suggested that the mitogenic ability of Ad 5 is probably associated with the same region of the viral genome that is required for rodent cell transformation (Graham et al., 1974). Ad 5 induced mitogenesis and transformation might be related as suggested from the experiments with SV40. The expression of the Ad 5 transforming region (early region 1A and 1B) during infection and after transformation has recently gained much attention by many researchers (Green et al., 1979; Lassam et al., 1979; Spector et al., 1979; van der Eb et al., 1979). In 293 cells, which are transformed with the left hand 11% of the Ad 5 genome (Graham et al., 1977), Spector et al., (1979) showed that both early region 1A RNA species (13s and 12s) and early region 1B RNA species (22s and 13s) were synthesised, and could be translated in vitro into proteins identical in size to those synthesised from these same regions during lytic infection. However, a 9s RNA species, observed during lytic infection,

was not found in these transformed cells. Each of the 1B RNAs can be assigned to polypeptides including a 15K and 52-58K species which probably correspond to the small and large "T" proteins. However, in other transformed cells the relationship between immunoprecipitated viral proteins, and those synthesised during lytic infection is very unclear (Lassam et al., 1979; Spector et al., 1979). In these experiments, several transformed rat cell lines were examined and in no case was any virus protein consistently immunoprecipitated. Thus, whilst expression of early regions 1A and 1B may be required to initiate transformation, they may not be required for the maintenance of the transformed phenotype. That is, Ad 5 modifies cells in some specific manner that allows transformation to be initiated (presumably by expression of regions 1A and 1B) but other factors are required in order that the transformed phenotype is selected and maintained. This cellular modification might be some cell cycle alteration such as aberrant DNA synthesis and mitosis.

Preliminary results reported in the previous chapter (Fig. 3.9 and Table 3.8) showed that Ad 5 not only induces G_1-arrested cells to undergo a round of DNA replication, but in some cases it causes an accumulation of cells with >4n (>G_2 diploid) DNA contents. That is, Ad 5 induces cells with aberrant DNA contents to form, some of which have polyploid DNA contents, as was reported for SV40 infected cells (see Section 4.1.1). Aside from these observations, there are other reports of adenovirus induced cell cycle abnormalities. McDougall et al., (1974) for

example, showed that Ad 12 induced substantial polyploid metaphases after infection of human cells (see Section 4.1 for details) and recently Murray et al., (1982) have shown with flow cytometry that Ad 5 causes marked cell cycle alterations in rodent cells, resulting in some cells with polyploid DNA contents and chromosome numbers. By analogy with SV 40, the induction of cells with aberrant DNA contents, such as polyploid cells, may be an important early step in the formation of transformed cells. It should be stressed however, that whilst some adenovirus induced transformants do have abnormal DNA contents and chromosome numbers, there are many examples which do not. Gallimore and Paraskeva (1979) compared a series of Ad 2 and Ad 12 transformed rat embryo brain cells with respect to their tumorogenicity, karyotype, and ability to synthesise T antigens. None of the Ad 2 transformed cell lines contained a normal diploid karyotype as determined by cytogenetic analysis and each transformant contained a heterogeneous cell population which mostly had pseudotriploid or pseudotetraploid DNA contents, but a small proportion were also diploid or pseudodiploid. Only 2/6 Ad 2 transformants were tumorogenic in nude mice and they all varied in their ability to synthesise T antigen detectable by immunofluorescence. Ad 12 transformants by contrast were all tumorogenic, nearly homogeneous in cell population, and 3/6 contained normal diploid chromosome numbers. Some were however aneuploid and tetrapolid. The cells also varied considerably in their ability to synthesise T antigen, but were all positive to some extent. Thus adenovirus induces transformed cells with a spectrum of karyotypes, although

the relationship between these karyotypic abnormalities and tumorogenicity remains unclear. Similar karyotype alterations in Ad 12 transformed rodent cells have been reported by Yamashita et al., (1980).

4.1.3 Ad 5 MUTANTS AND TRANSFORMATION

With the background discussed above, I decided to investigate adenovirus induced cell cycle alterations in rodent cells using the Ad 5 mutants discussed in the previous chapter; that is, ts 36, ts 125, dl 312 and dl 313. The defects in these mutants, and their transforming properties were discussed in Chapter 3 (in particular see Section 3.1). In addition, one other Ad 5 host-range (hr) defective, and transformation defective mutant was examined in this chapter for its ability to induce cell cycle aberrations. This mutant is hr 7 isolated by Harrison et al., (1977), which represents an example of a mutant from their complementation group II, which is equivalent to early region 1B. hr 7 has been accurately mapped by marker rescue to 6.1-9.0% of the Ad 5 genome (Frost and Williams, 1978). This complementation group is defective for T antigen synthesis (Harrison et al., 1977) and for transformation of rat embryo cells, rat embryo brain cells and baby rat kidney cells (Graham et al., 1978). hr 7 grows on 293 cells but not on Hela cells.

In this chapter DNA content analyses of mutant infected rodent cells was done using different techniques in order to investigate Ad 5 induced cell cycle alterations, and the results discussed in terms of transformation.

4.2 METHODS OF DNA CONTENT ANALYSIS

In this chapter three methods of DNA content analysis were used. Ultracentrifugation of extracted DNA and microspectrophotometry were described in previous chapters and were used for 2 experiments. However, for most experiments, the technique of flow cytometry was used for total cell cycle analysis in this chapter. This technique is extremely versatile in that the effects of viruses on the total cell growth cycle can be analysed rather than just effects on DNA replication (by isotope labeling) or metaphase chromosomes. Thus one can investigate many types of cell cycle alterations including premature reinitiation of DNA replication leading to abnormally high DNA contents and chromosome number. This technique is also extremely rapid compared to microspectrophotometry and classical cytogenetic methods. The principle and details of this technique are described below.

4.2.1 PRINCIPLE OF FLOW CYTOFLUORIMETRIC ASSAY OF DNA CONTENT

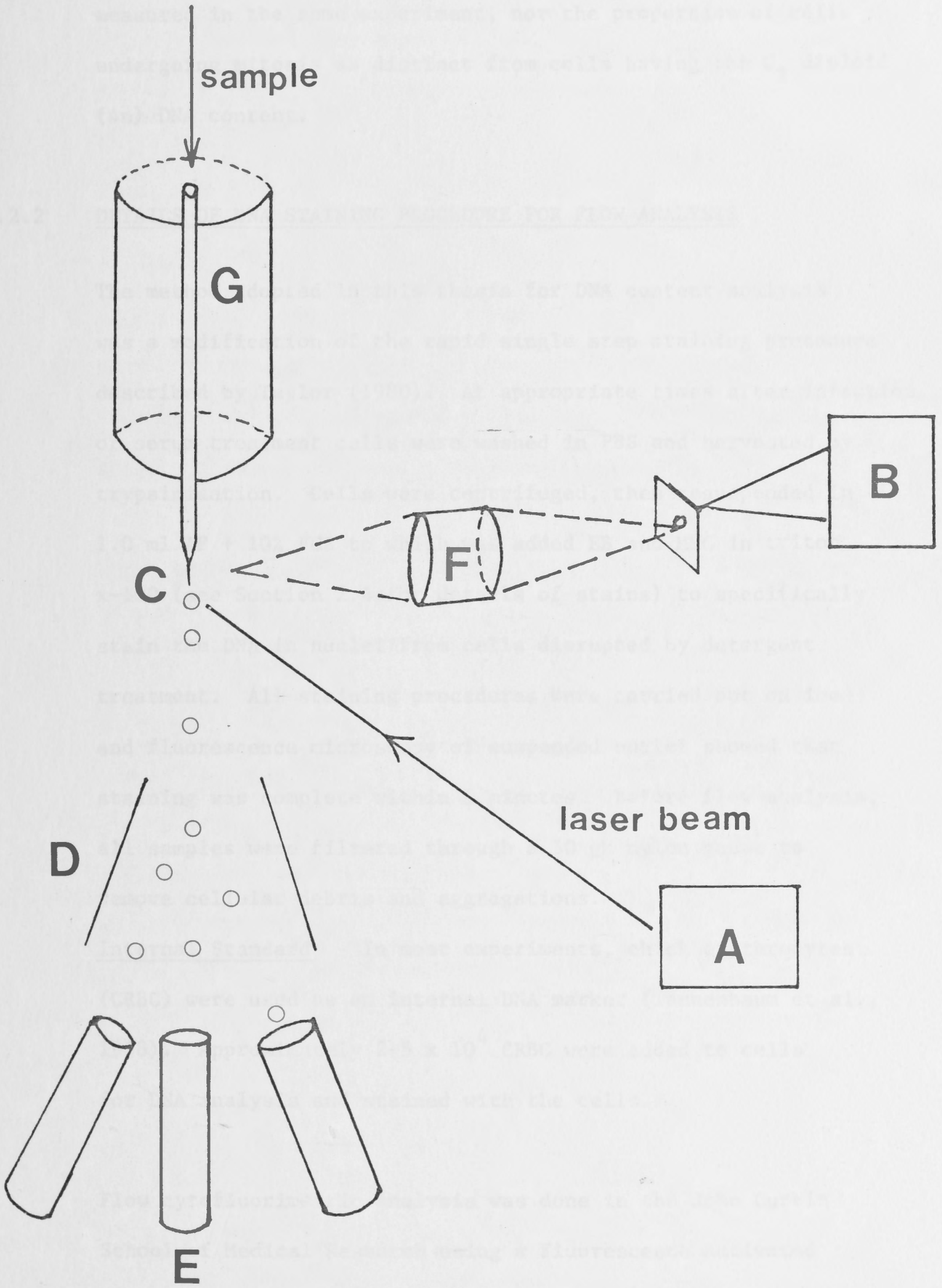
Particles for cytofluorimetric analysis (in this discussion, DNA) are stained with an appropriate fluorescent dye (fluorochrome) which for DNA is usually propidium iodide, chromomycin, ethidium bromide (EB), or mithramycin (MMC). Generally, in a flow system such as is illustrated in Fig. 4.1 (and which is available in Canberra), fluorescently stained particles are transported one-by-one under pressure

in a liquid medium (e.g. PBS) through an intense beam of light, the wavelength of which is specifically selected to excite the dye. The resulting fluorescence is measured and recorded using a pulse-height analyser and this fluorescence is proportional to the amount of substance to which the fluorochrome is specifically bound. For DNA analysis therefore, it is possible to analyse cells in different phases of the cell cycle (G_1, S, G_2 + M) by the amount of emitted fluorescence. The rate of fluorescent particle analysis is usually about 10^3 particles/second (Gray and Coffino, 1979). In addition, with the flow cytometer illustrated in Fig. 4.1, cells can be sorted (separated) on the basis of their fluorescence. The light source of most flow cytometers is an argon-ion laser (e.g. FACS IV, Becton - Dickinson, California, the instrument used for most of my work) but an exception is the Ortho flow cytometer (see Göhde, 1973), which uses an arc lamp (some experiments were done using this instrument).

Flow cytofluorimetric analysis of DNA is extremely advantageous to use as the staining procedures are simple and large numbers ($2\text{-}5 \times 10^4$) of fluorescently stained cells can be measured rapidly. Cell cycle analysis can then be done by "eye", or by using a computer program (see Milthorpe, 1980). Its major

FIGURE 4.1

Schematic representation of a flow sorter. Fluorescently stained cells in aqueous suspension are introduced into a sorter chamber (G). They traverse the chamber and emerge one-by-one in a liquid jet (C), which is broken into droplets which contain less than one cell per droplet. The fluorescent dye is excited by a laser beam (from source A). The resulting fluorescence is collected by a microscope objective (F) and projected through a filter (not shown) on to a photomultiplier (B). The photomultiplier and associated amplifier produce a voltage pulse whose height is proportional to the fluorescence intensity. The height of the voltage pulse is the criterion for cell sorting. Drops containing cells to be sorted (if desired) are deflected by an electric field generated by charged deflection plates (D). The sorted cells (or unsorted depending on the experiment) are then collected in tubes (E). For detailed explanation see Gray and Coffino (1979).


sample
G
C
F
B
laser beam
A
D
E

disadvantage is that changes in chromosome numbers cannot be measured in the same experiment, nor the proportion of cells undergoing mitosis as distinct from cells having the G_2 diploid (4n) DNA content.

4.2.2 DETAILS OF DNA STAINING PROCEDURE FOR FLOW ANALYSIS

The method adopted in this thesis for DNA content analysis was a modification of the rapid single step staining procedure described by Taylor (1980). At appropriate times after infection or serum treatment cells were washed in PBS and harvested by trypsinisation. Cells were centrifuged, then resuspended in 1.0 ml AP + 10% FCS to which was added EB and MMC in triton x-100 (see Section 2.4 for details of stains) to specifically stain the DNA in nuclei from cells disrupted by detergent treatment. All staining procedures were carried out on ice and fluorescence microscopy of suspended nuclei showed that staining was complete within 5 minutes. Before flow analysis, all samples were filtered through a 50 μM nylon gauze to remove cellular debris and aggregations.

Internal Standard In most experiments, chick erythrocytes (CRBC) were used as an internal DNA marker (Tannenbaum et al., 1978). Approximately 2-5 x 10^4 CRBC were added to cells for DNA analysis and stained with the cells.

Flow cytofluorimetric analysis was done in the John Curtin School of Medical Research using a Fluorescence Activated Cell Sorter (IV) (FACS IV, Becton-Dickinson, California) or at the Ludwig Institute for Cancer Research, Sydney, in

collaboration with Dr Ian Taylor using an ICP 22 pulse cytometer (Ortho instruments). The required excitation and emission wavelength for the fluorochromes were obtained by (i) prism rotation to select for the 457 nm excitation band and (ii) use of a 520 nm longpass filter for the emission spectrum. For experiments in which DNA content analysis was done in Sydney, cell cycle kinetic parameters (G_1, S, and G_2 + M) and coefficient of variance (CV) of the G_1 peak were computed from the DNA histograms by the method of Milthorpe (1980). This computer package excludes all cells with > G_2 diploid DNA content.

4.2.3 DNA CONTENT ANALYSIS OF DIFFERENT CELL TYPES

A preliminary experiment was done in order to observe the DNA content of cells known to have differing contents of cellular DNA. For this experiment growing mouse and rat cells were used (in AP + 10% FCS), as well as L929 mouse cells and rat cells treated with either hydroxyurea or colchicine. Hydroxyurea inhibits the enzyme ribonucleotide reductase, thus inhibiting DNA synthesis, and therefore causing a selective accumulation of cells at the G_1 phase of the cell cycle (Timson, 1975). This drug can therefore be used to define the position of the G_1 peak, relative to an internal DNA standard. Similarly, colchicine interferes with mitotic spindle formation by binding to tubulin (Taylor, 1965; Olmsted and Borisy, 1973; Garland, 1978) thus preventing tubulin polymerisation and causing a selective accumulation of G_2 diploid cells. It can therefore be used to define the G_2 peak position. The mouse

and rat cells used in the experiments in this thesis have 40 and 42 metaphase chromosomes respectively, whereas the continuous mouse L929 cell line is hypotetraploid (mode = 57) and has some structurally altered chromosomes. These cells were compared to determine whether abnormal chromosome structure and number give rise to an altered DNA fluorescence profile as determined by flow cytometry in the FACS IV. Results of these experiments (Fig. 4.2) showed (i) that hydroxyurea caused an accumulation of cells with a major peak position at channel 55, relative to the CRBC peak at channel 20; this therefore represents the G_1 peak; (ii) colchicine treated cultures had two major peaks, one at channel 55 and a second prominent peak representing cells blocked in G_2 + M at about channel 110, relative to the CRBC marker at 20; (iii) untreated rat and mouse cells both had main fluorescence peaks at channel 55 (relative to CRBC) and a smaller peak at 100-110, which therefore represent G_1 and G_2 diploid DNA peaks, and (iv) L929 cells had a main peak at channel 60 with an S phase shoulder leading to a second peak at channel 110, relative to CRBC at channel 7. The G_1 and G_2 peaks therefore have DNA contents about 3-fold higher than that of normal mouse cells. Thus cells with abnormal chromosomes and an abnormal ploidy give rise to an altered fluorescence distribution. As a result of these experiments, in the results presented in this thesis, the CRBC standard peak was set at about channel 20 thus establishing the G_1 DNA peak at about channels 50-55. All aberrant DNA contents were measured relative to these two peak positions.

FIGURE 4.2

Flow cytometry determined DNA histograms of different types of cells, including cells treated with hydroxyurea and colchicine. Cells were harvested and the DNA stained with ethidium bromide and mithramycin as described in Section 4.2.2. Cells in this experiment were analysed for DNA content* using the FACS IV in Canberra. The cell cycle kinetic parameter analysis is not at present operable, so this experiment shows only qualitative differences in DNA content, unlike those experiments analysed in Sydney using the computer package developed by Milthorpe (1980).

(A) Rat cells plus 5mM hydroxyurea for 2 days;

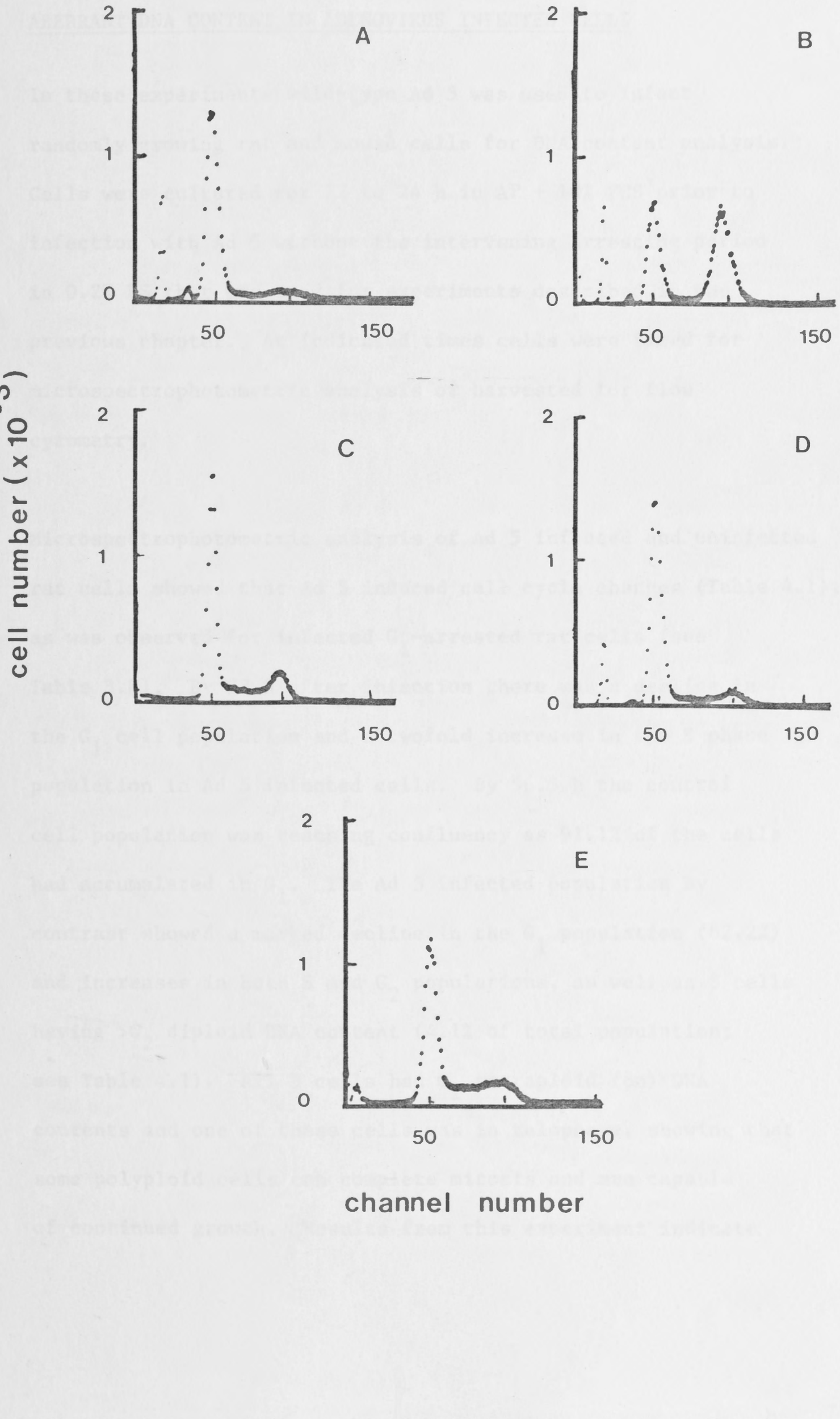
(B) Rat cells plus 1 μM colchicine for 2 days;

(C) Untreated rat cells (no CRBC standard);

(D) Untreated mouse cells;

(E) L929 cells

* Figures represent profiles photographed directly from the visual display on the FACS IV.


A
B
C
D
E
2
1
0
50
150
cell number (x 10-3)
channel number

4.3 RESULTS

4.3.1. ABERRANT DNA CONTENT IN ADENOVIRUS INFECTED CELLS

In these experiments wild-type Ad 5 was used to infect randomly growing rat and mouse cells for DNA content analysis. Cells were cultured for 12 to 24 h in AP + 10% FCS prior to infection with Ad 5 without the intervening arresting period in 0.2% BS that was used for experiments described in the previous chapter. At indicated times cells were fixed for microspectrophotometric analysis or harvested for flow cytometry.

Microspectrophotometric analysis of Ad 5 infected and uninfected rat cells showed that Ad 5 induced cell cycle changes (Table 4.1), as was observed for infected G_1-arrested rat cells (see Table 3.8). By 22 h after infection there was a decline in the G_1 cell population and a twofold increase in the S phase population in Ad 5 infected cells. By 56.5 h the control cell population was reaching confluency as 91.1% of the cells had accumulated in G_1. The Ad 5 infected population by contrast showed a marked decline in the G_1 population (62.2%) and increases in both S and G_2 populations, as well as 3 cells having $>G_2$ diploid DNA content (4.1% of total population; see Table 4.1). All 3 cells had G_2 tetraploid (8n) DNA contents and one of these cells was in telophase, showing that some polyploid cells can complete mitosis and are capable of continued growth. Results from this experiment indicate

TABLE 4.1

DNA Contents of Ad 5 infected rat cells measured by microspectrophotometry [b] and autoradiography [a]

DNA content (extinction score x 10^{-2}) [c]	Approximate stage of cell cycle	Percentage of cells			
		22 h		56.5 h	
		Mock infected	Ad 5 infected	Mock infected	Ad 5 infected
40-69	G_1	85.7	80.2	91.1	62.2
70-99	S	3.3	6.6	4.4	11.2
100-129	G_2	11.0	12.1	4.4	22.4
129	$> G_2$	0	1.1	0	4.1

a 1,000 cells were scored for the presence of nuclear grains ($[^3H]$ thymidine incorporation) to estimate the proportion of cells in S phase

b 90 cells without nuclear grains were analysed by microspectrophotometry to determine the relative proportions of cells with G_1, G_2 + M, or $>G_2$ diploid DNA contents

c Arbitrary measure of feulgen dye excitation to measure DNA contents

that Ad 5 induces an abnormal mitogenic response in growing cells as well as suggesting that an aberrant mitosis takes place in a proportion of infected cells, and a second round of DNA replication occurs. These cell cycle abnormalities are therefore not a unique feature of G_1-arrested cells.

A second experiment was done using flow cytometry of $1\text{-}2 \times 10^4$ cells from infected and uninfected cultures. Once again, randomly growing cells were used. The results of this experiment (Fig. 4.3) also showed substantial cell cycle alterations by Ad 5 infection. 1 day after infection there was a small decline in G_1 cells and a small increase in S phase cells relative to uninfected controls. By 2.5 days after infection this trend was even more pronounced and there was a sharp increase in G_2 (+M) cells (25.7% compared to 10.4% for control), and also many cells showing $> G_2$ diploid DNA contents which by 5 days after infection had increased to about 30% of the total population. The insets in Fig. 4.3 showing computed cell cycle kinetic parameters was calculated using the computer package of Milthorpe (1980) which excludes cells having $> G_2$ diploid (4n) DNA contents. As a result, this experiment is tabulated in Table 4.2 where the number of cells analysed by computer (into G_1, S and G_2 + M) is substracted from the total number of cells examined. The difference therefore represents those cells having $> G_2$ diploid DNA contents. This tabulation clearly shows that Ad 5 induces marked abnormalities in mitosis leading to an accumulation of cells with > 4n DNA contents.

FIGURE 4.3

DNA content analysis of Ad 5 infected cycling rat cells. Cells were treated as described in the text and processed for flow cytometry as described in Section 4.2.2. This experiment was carried out using the Ortho flow cytometer in Sydney.

(a) MOCK 1 day (b) Ad 5 infected, 1 day (43% P antiserum positive by 48 h after infection

(c) MOCK 2.5 days (d) Ad 5 infected, 2.5 days (28% P antiserum positive by 48 h).

(e) MOCK 5 days (f) Ad 5 infected, 5 days (60% P antiserum positive by 48 h). The peak at channel 18 is the CRBC marker.

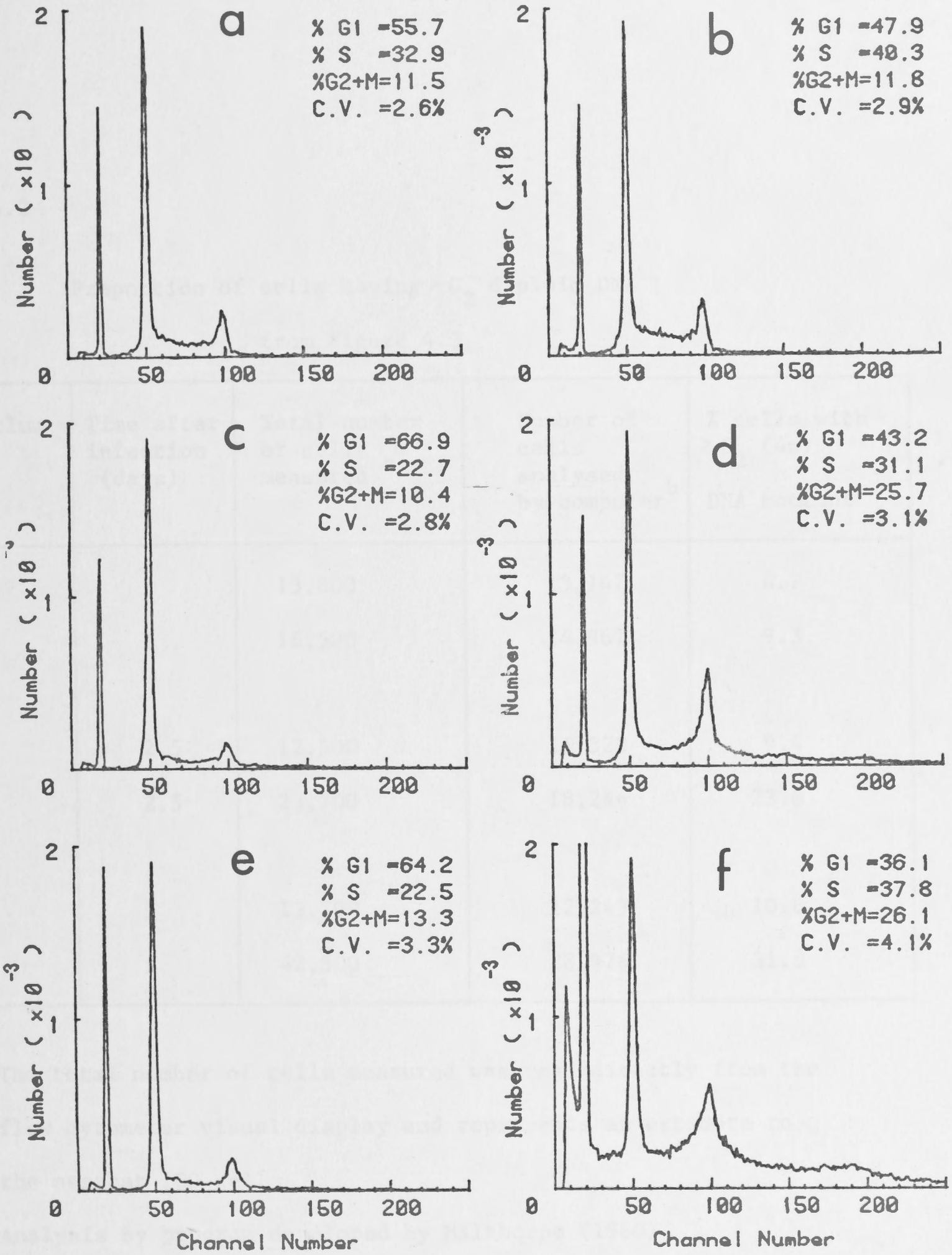

a
% G1 =55.7
% S =32.9
%G2+M=11.5
C.V. =2.6%
b
% G1 =47.9
% S =40.3
%G2+M=11.8
C.V. =2.9%
c
% G1 =66.9
% S =22.7
%G2+M=10.4
C.V. =2.8%
d
% G1 =43.2
% S =31.1
%G2+M=25.7
C.V. =3.1%
e
% G1 =64.2
% S =22.5
%G2+M=13.3
C.V. =3.3%
f
% G1 =36.1
% S =37.8
%G2+M=26.1
C.V. =4.1%
Number (x10 -3)
Channel Number
0
50
100
150
200
1
2

TABLE 4.2

Proportion of cells having > G_2 diploid DNA

from Figure 4.3

Inoculum	Time after infection (days)	Total number of cells measured [a]	Number of cells analysed by computer [b]	% cells with > G_2 (4n) DNA content
MOCK	1	13,800	13,147	4.7
Ad 5	1	16,500	14,967	9.3
MOCK	2.5	12,500	11,323	9.4
Ad 5	2.5	23,700	18,246	23.0
MOCK	5	13,700	12,243	10.6
Ad 5	5	42,500	28,976	31.8

a The total number of cells measured was read directly from the flow cytometer visual display and represents an estimate to the nearest 100 cells

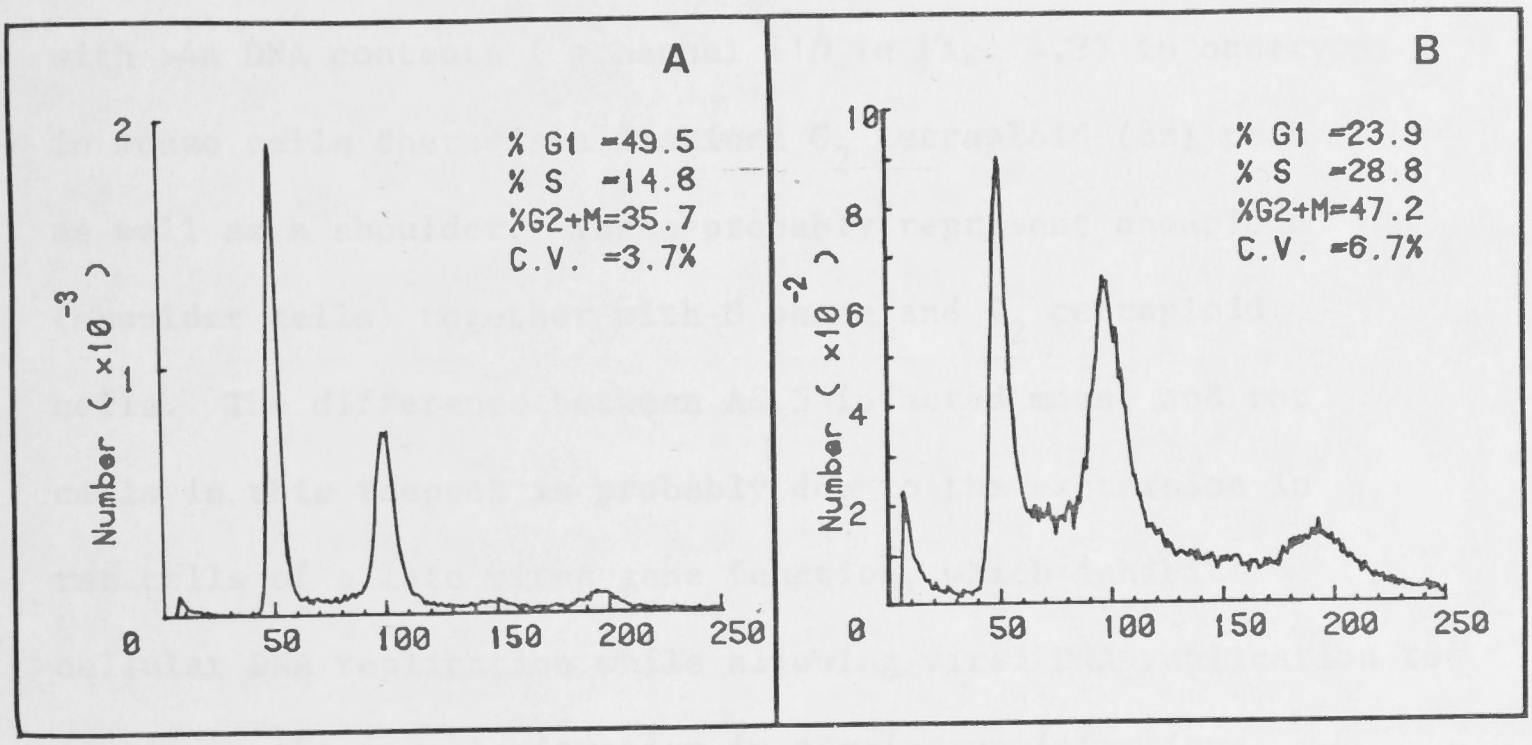
b Analysis by program developed by Milthorpe (1980)

FIGURE 4.4

DNA histograms of Ad 5 infected and uninfected mouse cells determined by flow cytometry. Cells were infected or mock infected as described in Section 2.1.4 and 2 days later were harvested and stained for flow cytometric analysis of DNA content. This experiment was done using the Ortho flow cytometer in Sydney. For details of staining procedure see Section 4.2.2.

(A) MOCK infected cells

(B) Ad 5 infected cells


A
% G1 =49.5
% S =14.8
%G2+M=35.7
C.V. =3.7%
Number (x10 -3)
2
1
0
50
100
150
200
250
B
% G1 =23.9
% S =28.8
%G2+M=47.2
C.V. =6.7%
Number (x10 -2)
10
8
6
4
2
0
50
100
150
200
250
channel number

A similar experiment using flow cytometry was done using Ad 5 infected mouse cells. Once again, Ad 5 caused a decline in G_1 cells and an increase in S and G_2 cells relative to a control population (Fig. 4.4). In addition, many cells were observed with DNA contents >4n (18% in control; 33% in Ad 5 infected cells), suggesting again that in virus infected mouse cells an aberrant mitosis or complete absence of mitosis occurs. In contrast to infected rat cells where a shoulder of cells with >4n DNA contents (>channel 110 in Fig. 4.3) is observed, in mouse cells there is a distinct G_2 tetraploid (8n) peak as well as a shoulder. These probably represent aneuploid (shoulder cells) together with S phase and G_2 tetraploid cells. The difference between Ad 5 infected mouse and rat cells in this respect is probably due to the expression in rat cells of a late virus gene function, which inhibits cellular DNA replication while allowing viral DNA replication to continue, the normal situation in permissive infections. Expression of late genes is greater in rat cells than in mouse cells as they are more permissive for Ad 5 replication (compare Gallimore (1974) with Younghusband et al., (1979)). This is discussed in detail later in this chapter.

4.3.2. IS ADENOVIRUS GENE EXPRESSION REQUIRED FOR INDUCTION OF AN ABERRANT CELL CYCLE IN RAT CELLS?

Results presented in the previous chapter (see Section 3.3.2) showed that Ad 5 induced cellular DNA replication required the expression of at least one Ad 5 gene, and probably from early region 1A (see Sections 3.3.5 and discussion in

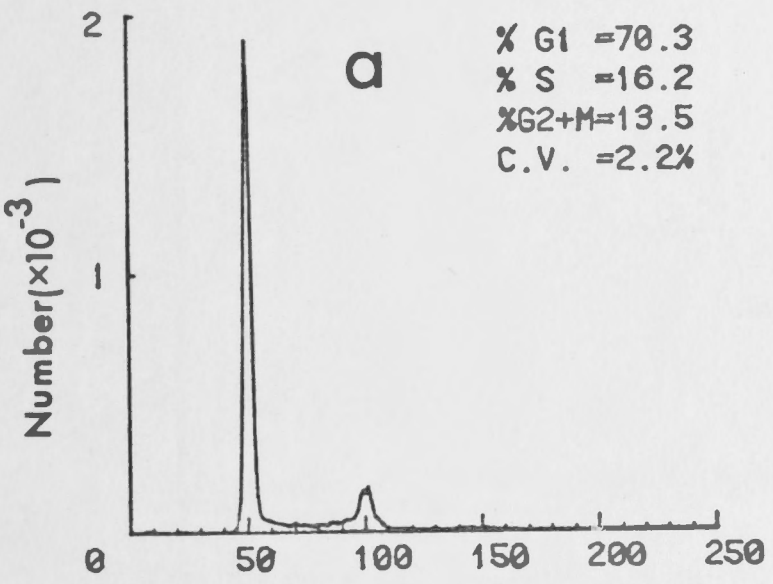
FIGURE 4.5

Multiplicity dependence of Ad 5 induced changes in cellular DNA content as determined by flow cytometry. Randomly growing rat cells were infected with different amounts of an Ad 5 inoculum then incubated for 2 days at 36.5°C. At this time cells were harvested and stained with EB and MMC for analysis by flow cytometry. For details of infection, staining and harvesting, see Sections 2.1.4, 4.2.2, and the legend to Fig. 3.9. This experiment was done using the Ortho flow cytometer in Sydney.

(a) MOCK infected; (b) 1.0 iu/cell Ad 5

(c) 5.0 iu/cell Ad 5; (d) 10.0 iu/cell Ad 5

(e) 25.0 iu/cell Ad 5


a
% G1 =70.3
% S =16.2
%G2+M=13.5
C.V. =2.2%
Number(×10-3)
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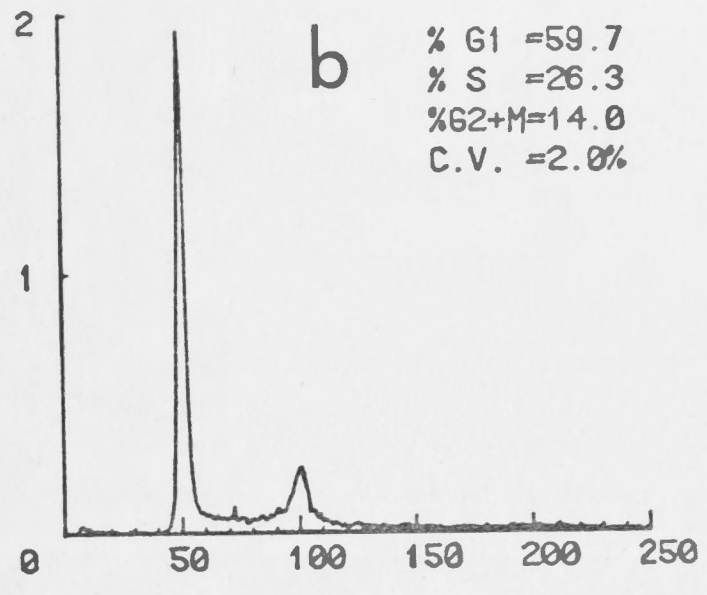
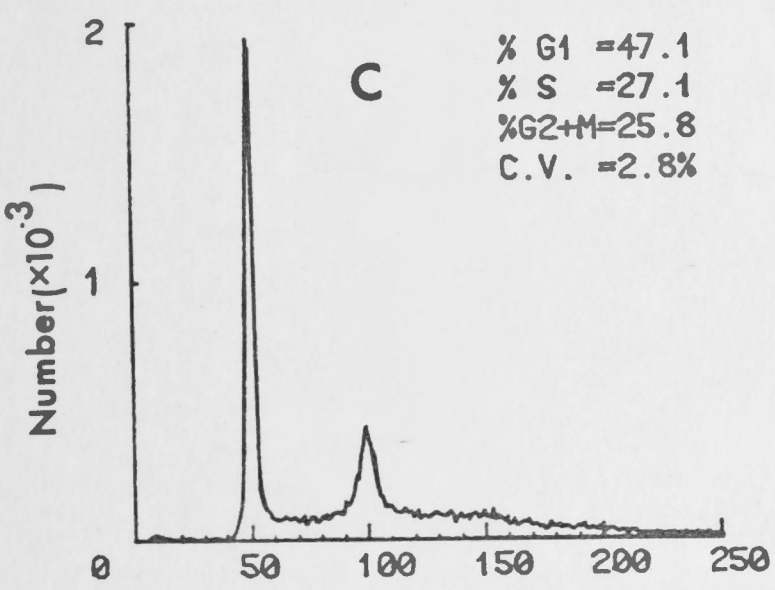
50
100
150
200
250


b
% G1 =59.7
% S =26.3
%G2+M=14.0
C.V. =2.0%
2
1
0
50
100
150
200
250


c
% G1 =47.1
% S =27.1
%G2+M=25.8
C.V. =2.8%
Number(×10-3)
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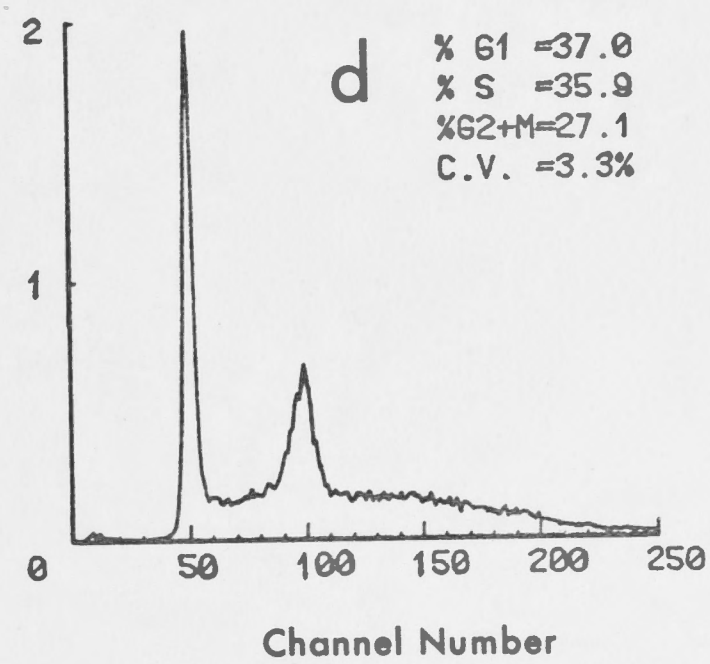
1
0
50
100
150
200
250


d
% G1 =37.0
% S =35.9
%G2+M=27.1
C.V. =3.3%
2
1
0
50
100
150
200
250
Channel Number

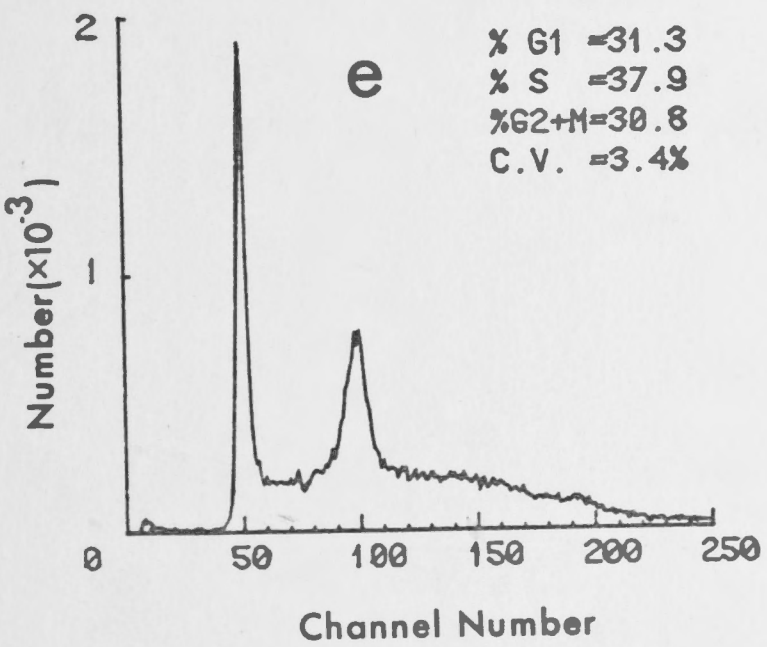

e
% G1 =31.3
% S =37.9
%G2+M=30.8
C.V. =3.4%
Number(×10-3)
2
1
0
50
100
150
200
250
Channel Number

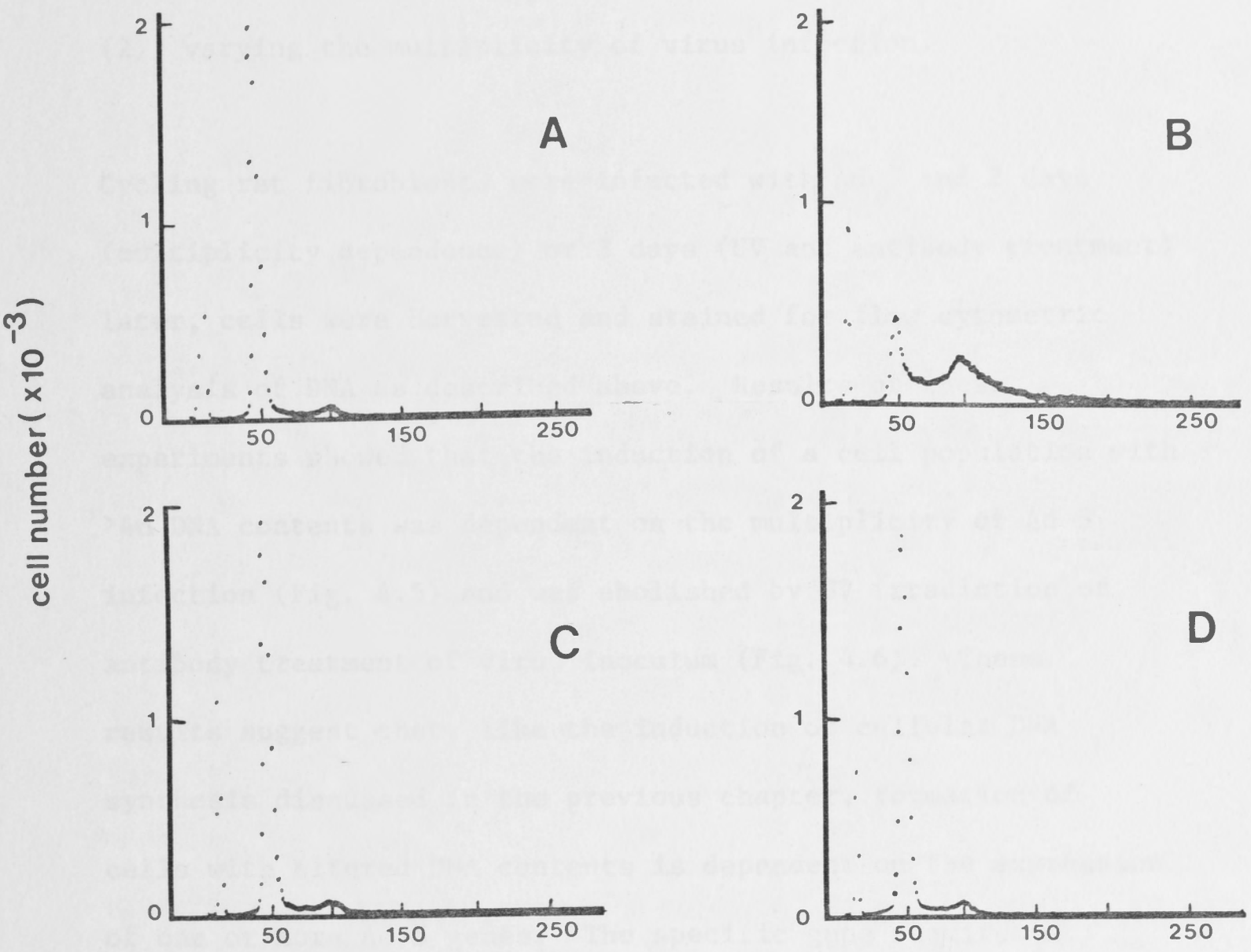
FIGURE 4.6

Effect of UV irradiation and antiserum treatment of the inoculum on changes in cellular DNA content induced by Ad 5 in rat cells. Cells were infected with Ad 5 wild-type, UV irradiated Ad 5, or Ad 5 pretreated with antibody,and incubated for 3 days at 36.5^{o}C. Cells were then harvested and stained for flow cytometric analysis of DNA content (for more details see Section 4.2.2 and the legend to Fig. 4.2). This experiment was done using the FACS IV in Canberra.

(A) MOCK infected rat cells; (B) Ad 5 infected control

(C) Ad 5 infected cells after incubation of virus with antiserum (1:5) to Ad 5 at 37^{o}C for 0.5 h

(D) Ad 5 infected, pretreated with UV irradiation (800 μ Wcm^{-2} at 0^{o}C for 0.5 h).


A
B
C
D
cell number (x10^{-3})
2
1
0
50
150
250
channel number

Section 3.4). The requirement for Ad 5 gene expression for altered cell cycle kinetics is tested in this section by analysing the effect of (1) UV irradiation or antibody treatment of Ad 5 inoculum before infection, and (2) varying the multiplicity of virus infection.

Cycling rat fibroblasts were infected with Ad 5 and 2 days (multiplicity dependence) or 3 days (UV and antibody treatment) later, cells were harvested and stained for flow cytometric analysis of DNA as described above. Results of these experiments showed that the induction of a cell population with >4n DNA contents was dependent on the multiplicity of Ad 5 infection (Fig. 4.5) and was abolished by UV irradiation or antibody treatment of virus inoculum (Fig. 4.6). These results suggest that, like the induction of cellular DNA synthesis discussed in the previous chapter, formation of cells with altered DNA contents is dependent on the expression of one or more Ad 5 genes. The specific gene requirement for this phenomenon is investigated in detail with the use of Ad 5 mutants in the following sections.

4.3.3 EFFECT OF SPECIFIC MUTATIONS IN THE Ad 5 GENOME ON THE FORMATION OF ALTERED DNA CONTENTS IN VIRUS INFECTED RAT CELLS

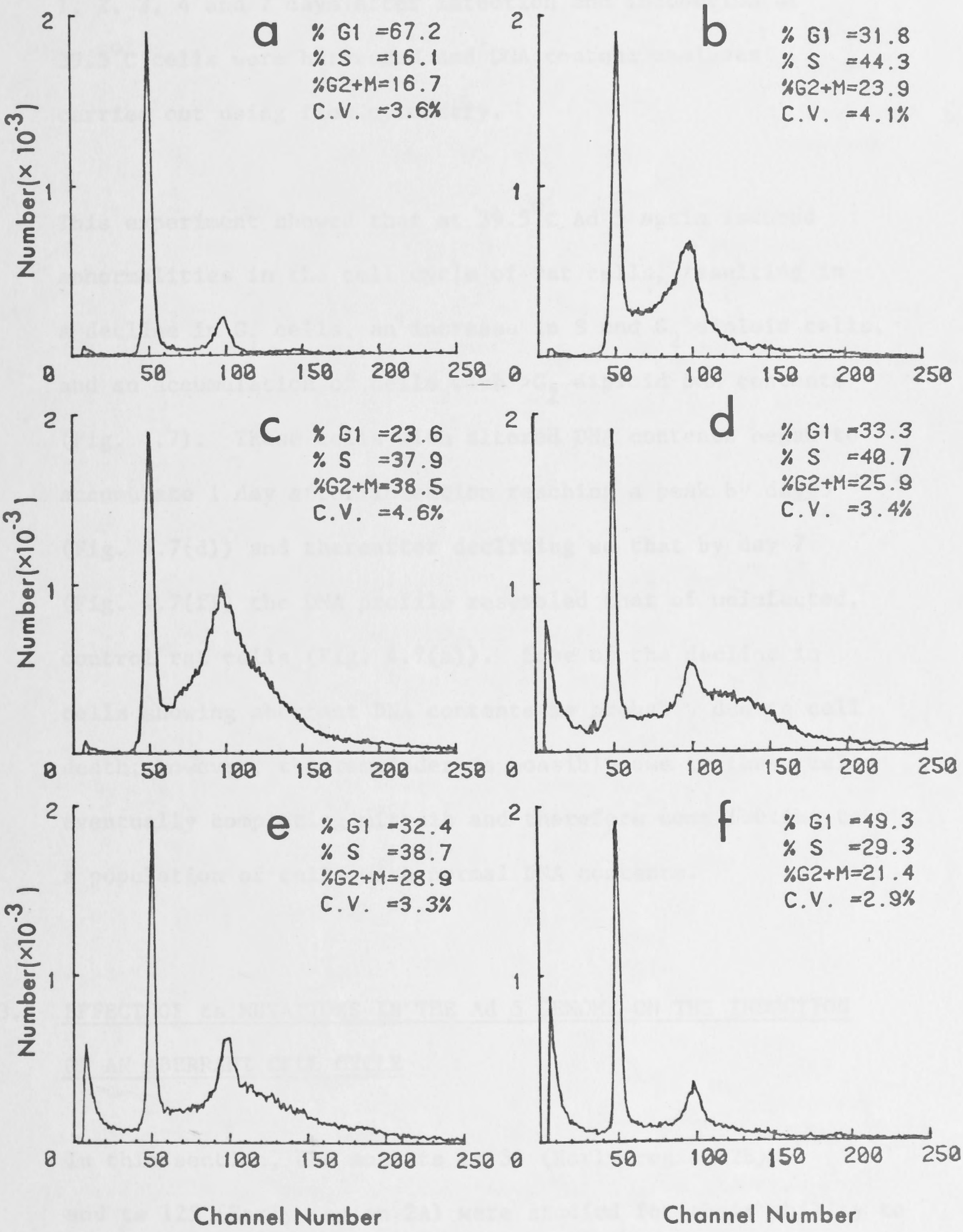
4.3.3.1 KINETICS OF FORMATION OF CELLS WITH ALTERED DNA CONTENTS BY Ad 5 WILD TYPE INFECTION AT 39.5°C

As a control for the ts Ad 5 mutants (ts 36 and ts 125), Ad 5 wild-type was assayed for its ability to cause cells with aberrant DNA contents over several days after infection

FIGURE 4.7

DNA histograms of rat cells infected with Ad 5 and incubated at 39.5°C (the nonpermissive temperature for ts mutant replication). Cells were infected with Ad 5 and after incubation at 39.5°C for indicated times they were harvested and stained with DNA fluorochromes for analysis by flow cytometry. This experiment was also done using the Ortho flow cytometer in Sydney. For details of staining procedure refer to Section 4.2.2.

(a) MOCK infected cells, 2 days; (b) Ad 5 infected cells, 1 day; (c) Ad 5 infected cells, 2 days; (d) Ad 5 infected cells, 3 days; (e) Ad 5 infected cells, 4 days; (f) Ad 5 infected cells, 7 days.


a
% G1 =67.2
% S =16.1
%G2+M=16.7
C.V. =3.6%
b
% G1 =31.8
% S =44.3
%G2+M=23.9
C.V. =4.1%
c
% G1 =23.6
% S =37.9
%G2+M=38.5
C.V. =4.6%
d
% G1 =33.3
% S =40.7
%G2+M=25.9
C.V. =3.4%
e
% G1 =32.4
% S =38.7
%G2+M=28.9
C.V. =3.3%
f
% G1 =49.3
% S =29.3
%G2+M=21.4
C.V. =2.9%
Number(x 10-3)
Number(x10-3)
Number(x10-3)
0
1
2
50
100
150
200
250
Channel Number
Channel Number

at the nonpermissive temperature for ts mutant replication (39.5°C). Cycling rat cells were infected with Ad 5 then 1, 2, 3, 4 and 7 days after infection and incubation at 39.5°C cells were harvested and DNA content analyses carried out using flow cytometry.

This experiment showed that at 39.5°C Ad 5 again induced abnormalities in the cell cycle of rat cells, resulting in a decline in G_1 cells, an increase in S and G_2 diploid cells, and an accumulation of cells with $>G_2$ diploid DNA contents (Fig. 4.7). These cells with altered DNA contents began to accumulate 1 day after infection reaching a peak by day 3 (Fig. 4.7(d)) and thereafter declining so that by day 7 (Fig. 4.7(f)) the DNA profile resembled that of uninfected, control rat cells (Fig. 4.7(a)). Some of the decline in cells showing aberrant DNA contents is probably due to cell death; however, the remainder is possibly due to these cells eventually completing mitosis and therefore contributing to a population of cells with normal DNA contents.

4.3.3.2 EFFECT OF ts MUTATIONS IN THE Ad 5 GENOME ON THE INDUCTION OF AN ABERRANT CELL CYCLE

In this section, the mutants ts 36 (Early region 2B) and ts 125 (Early region 2A) were studied for their ability to induce abnormalities in the cell cycle of rat cells at

permissive (32.5°C) and nonpermissive (39.5°C) temperatures for virus DNA replication. Mutant ts 36 has a mutation in gene N and ts 125 has a mutation in the region coding for the DNA binding protein (see Chapter 3, in particular Section 3.1, for details of the properties of these mutants). Mutant ts 36 is defective for transformation of rat fibroblasts at 38.5°C but behaves like wild-type Ad 5 at 32.5°C, and ts 125 transforms rat cells with a higher frequency than wild-type Ad 5 at all temperatures (Section 3.1).

At 39.5°C both ts 125 (Fig. 4.8) and ts 36 (Fig. 4.9) induced cell cycle kinetic alterations in which G_1 cells declined, and S, G_2 and cells with $>G_2$ diploid DNA contents increased relative to uninfected controls. Both mutants were tested in the same experiment and the proportion of P antiserum positive cells was determined to be 25-30% for ts 125 (measured at 36.5°C) and 35-40% for ts 36. As was observed for wild-type Ad 5, both ts 36 and ts 125 induced cell cycle alterations which were detectable by 1 day after infection, and were maximal at 3-4 days after infection (Fig. 4.8(d) for ts 125; Fig. 4.9(d) for ts 36), but had declined by day 7.

In contrast to the result with wild-type Ad 5, both ts 125 and ts 36 induced the formation of a distinct population of cells with an 8n DNA content rather than a shoulder of cells having DNA contents between 4n and 8n. This 8n peak was particularly pronounced in ts 125 infected cells (Fig. 4.8). In ts 36 infected cultures 25-30% showed aberrant DNA contents

FIGURE 4.8

Induction of an aberrant cell cycle in rat cells infected with ts 125 at the nonpermissive temperature (39.5°C). Cells were infected with mutant ts 125 and then incubated at 39.5°C for different periods of time up to 7 days after infection. At indicated times (see below) cells were harvested and stained for flow cytometric analysis of DNA content. This experiment was done using the Ortho flow cytometer in Sydney.

(a) MOCK infected; (b) ts 125 infected, 1 day;

(c) ts 125 infected, 2 days; (d) ts 125 infected,3 days;

(e) ts 125 infected, 4 days; (f) ts 125 infected, 7 days.

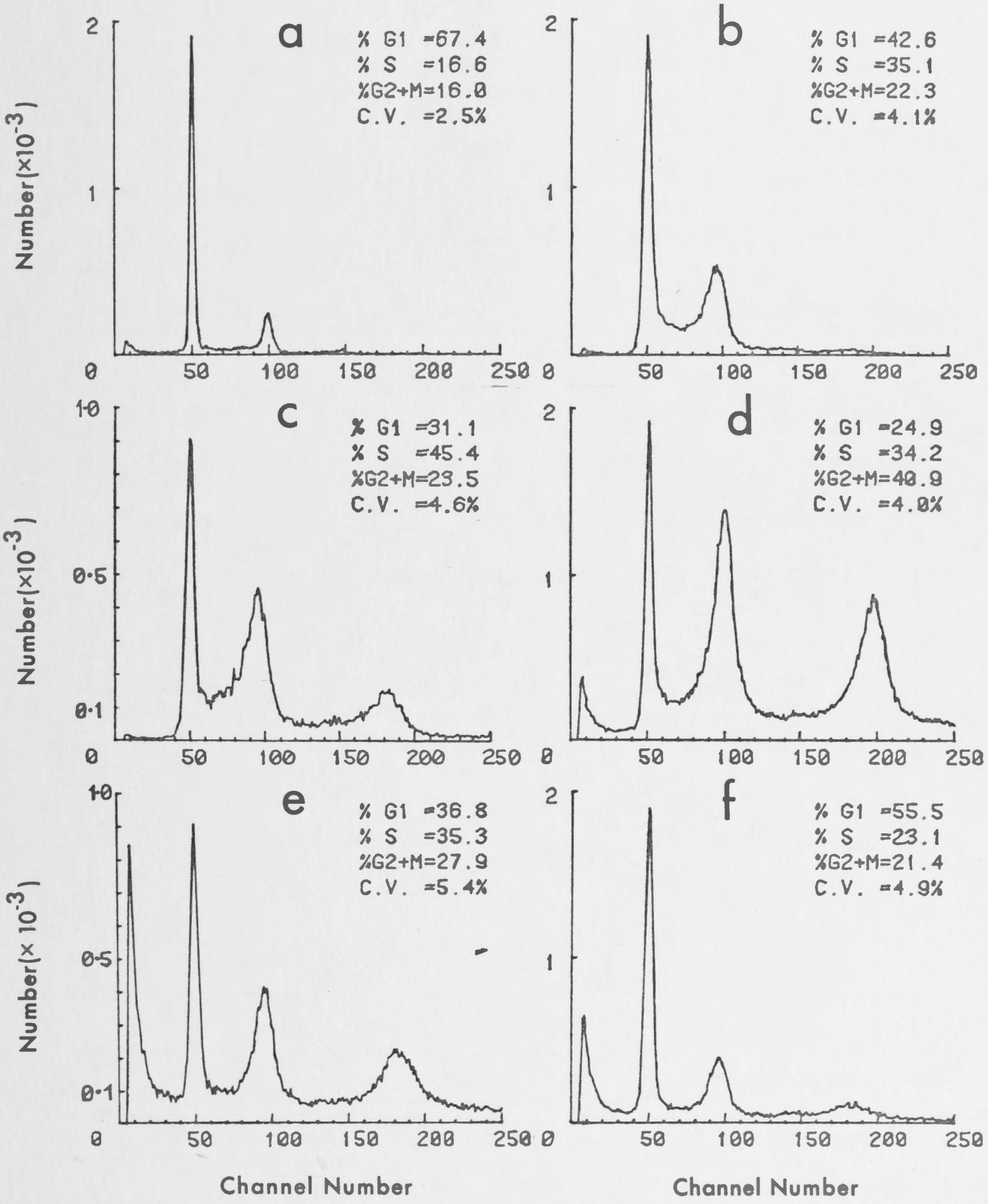

a
% G1 =67.4
% S =16.6
%G2+M=16.0
C.V. =2.5%
b
% G1 =42.6
% S =35.1
%G2+M=22.3
C.V. =4.1%
c
% G1 =31.1
% S =45.4
%G2+M=23.5
C.V. =4.6%
d
% G1 =24.9
% S =34.2
%G2+M=40.9
C.V. =4.0%
e
% G1 =36.8
% S =35.3
%G2+M=27.9
C.V. =5.4%
f
% G1 =55.5
% S =23.1
%G2+M=21.4
C.V. =4.9%
Number(×10-3)
Number(× 10-3)
Channel Number
Channel Number

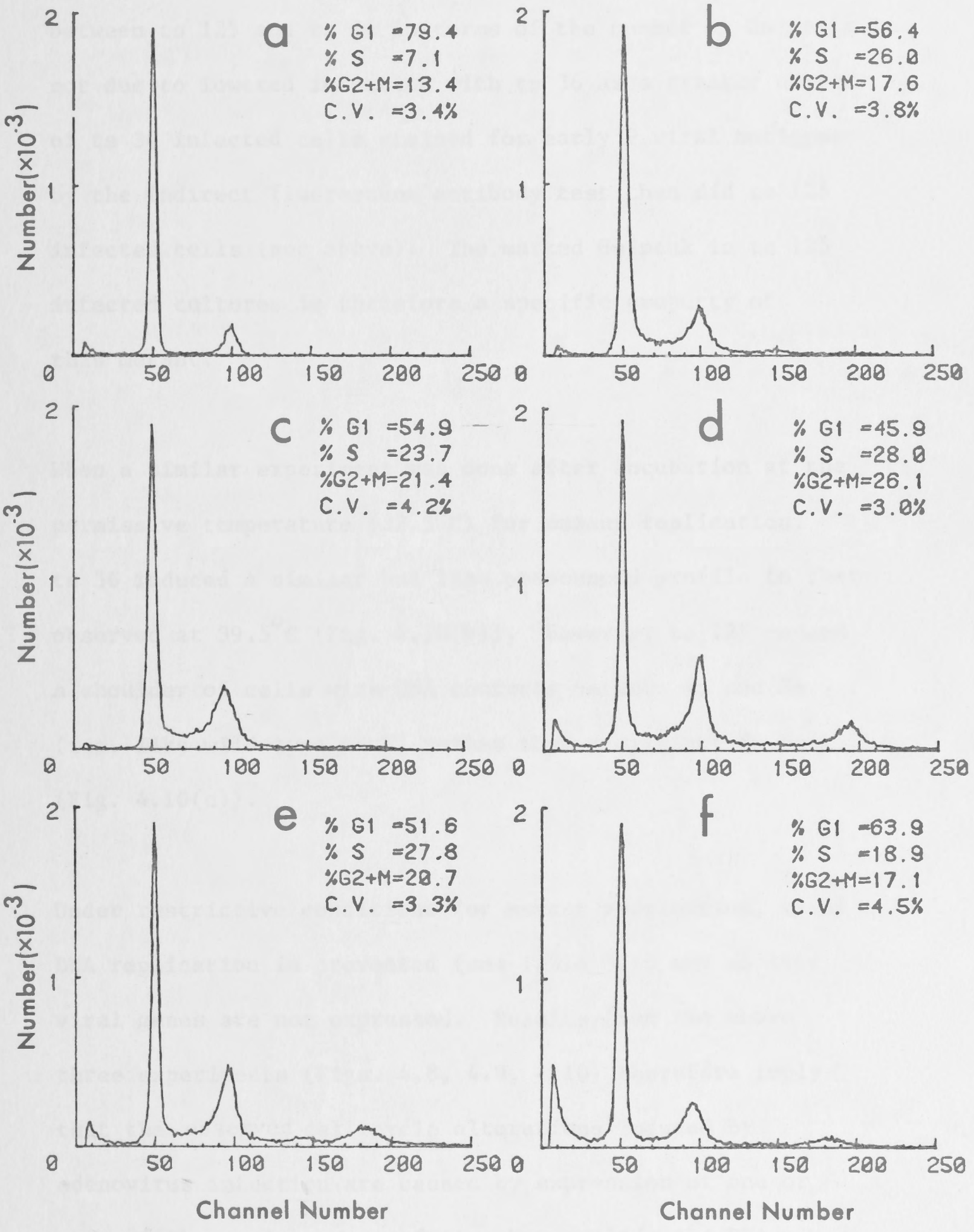
FIGURE 4.9

Induction of an aberrant cell cycle in rat cells infected with ts 36 at the nonpermissive temperature (39.5°C). Cells were infected with ts 36 and then incubated at the nonpermissive temperature for mutant replication (39.5°C). At indicated times cells were harvested and stained for flow cytometry. For details of staining procedure refer to Section 4.2.2. This experiment was done using the Ortho flow cytometer in Sydney.

(a) MOCK infected; (b) ts 36 infected, 1 day;

(c) ts 36 infected, 2 days (d) ts 36 infected, 3 days;

(e) ts 36 infected, 4 days; (f) ts 36 infected, 7 days.


a
% G1 =79.4
% S =7.1
%G2+M=13.4
C.V. =3.4%
b
% G1 =56.4
% S =26.0
%G2+M=17.6
C.V. =3.8%
c
% G1 =54.9
% S =23.7
%G2+M=21.4
C.V. =4.2%
d
% G1 =45.9
% S =28.0
%G2+M=26.1
C.V. =3.0%
e
% G1 =51.6
% S =27.8
%G2+M=20.7
C.V. =3.3%
f
% G1 =63.9
% S =18.9
%G2+M=17.1
C.V. =4.5%
Number(×10⁻³)
Channel Number
0
50
100
150
200
250
1
2

whereas 50% or more showed this shift to $>G_2$ diploid DNA contents in ts 125 infected cultures. The difference between ts 125 and ts 36 in terms of the number of 8n cells is not due to lowered infection with ts 36 as a greater number of ts 36 infected cells stained for early P viral antigens by the indirect fluorescent antibody test than did ts 125 infected cells (see above). The marked 8n peak in ts 125 infected cultures is therefore a specific property of this mutant.

—

When a similar experiment was done after incubation at the permissive temperature (32.5°C) for mutant replication, ts 36 induced a similar but less pronounced profile to that observed at 39.5°C (Fig. 4.10(b)). However, ts 125 caused a shoulder of cells with DNA contents between 4n and 8n (i.e. like wild-type Ad 5) rather than a distinct 8n peak (Fig. 4.10(c)).

Under restrictive conditions for mutant replication, viral DNA replication is prevented (see Table 3.4) and so late viral genes are not expressed. Results from the above three experiments (Figs. 4.8, 4.9, 4.10) therefore imply that the observed cell cycle alterations induced by adenovirus infection are caused by expression of one or more of the early gene products, but exclude the DNA binding protein and the gene N product from being responsible. The results with these mutants also suggest that when late genes are not expressed distinct tetraploidy occurs, but an abortive second round of cellular

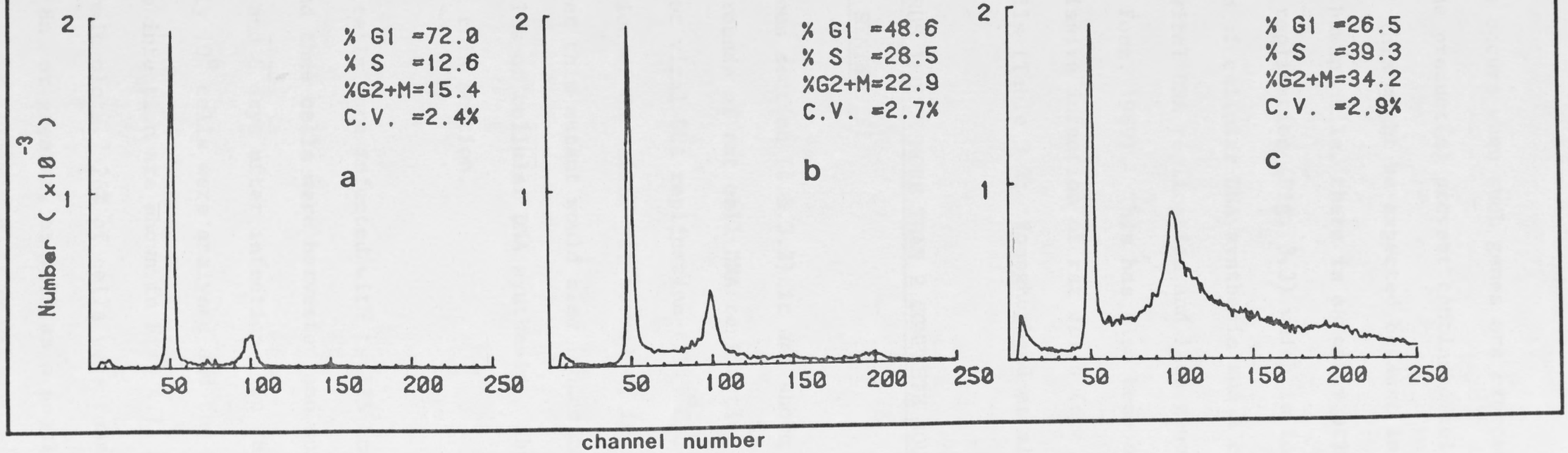
FIGURE 4.10

DNA histograms of rat cells infected with ts 36 and ts 125 under permissive conditions for mutant replication (32.5°C). Cells were infected with mutants, or mock infected, then incubated at 32.5°C for 3 days. Cells were then harvested and stained for DNA content analysis by flow cytometry. This experiment was done using the Ortho flow cytometer in Sydney. For details of staining procedure see Section 4.2.2.

(a) MOCK infected cells; 23% P antiserum positive;

(b) ts 36 infected cells,

(c) ts 125 infected cells, 32% P antiserum positive.


% G1 =72.0
% S =12.6
%G2+M=15.4
C.V. =2.4%
a
% G1 =48.6
% S =28.5
%G2+M=22.9
C.V. =2.7%
b
% G1 =26.5
% S =39.3
%G2+M=34.2
C.V. =2.9%
c
Number (x10^-3)
1
2
50
100
150
200
250
channel number

DNA synthesis occurs when such genes are expressed. Thus some late gene product(s) prevent continued cell cycle alterations. This might be expected because in a permissive infection of human cells, there is an early stimulation of cellular DNA replication (Fig. 3.3) which is followed by an inhibition of cellular DNA synthesis and a concomitant increase in viral DNA replication and late gene expression (Ledinko and Fong, 1969). This has also been observed for the semipermissive infection of rat cells (see Table 3.2) and mouse cells (Table 3.2; Younghusband et al., 1979).

4.3.3.3 DOES ADENOVIRUS INDUCE MORE THAN 2 COMPLETE ROUNDS OF CELLULAR DNA SYNTHESIS?

In the previous section (4.3.3.2) it was shown that ts 125 induced two rounds of rat cell DNA replication under restrictive conditions for viral DNA replication (39.5^{o}C), resulting in an accumulation of 8n cells. It seemed of interest therefore to ask whether this mutant would also induce more than two complete cycles of cellular DNA synthesis. This possibility was tested in this section.

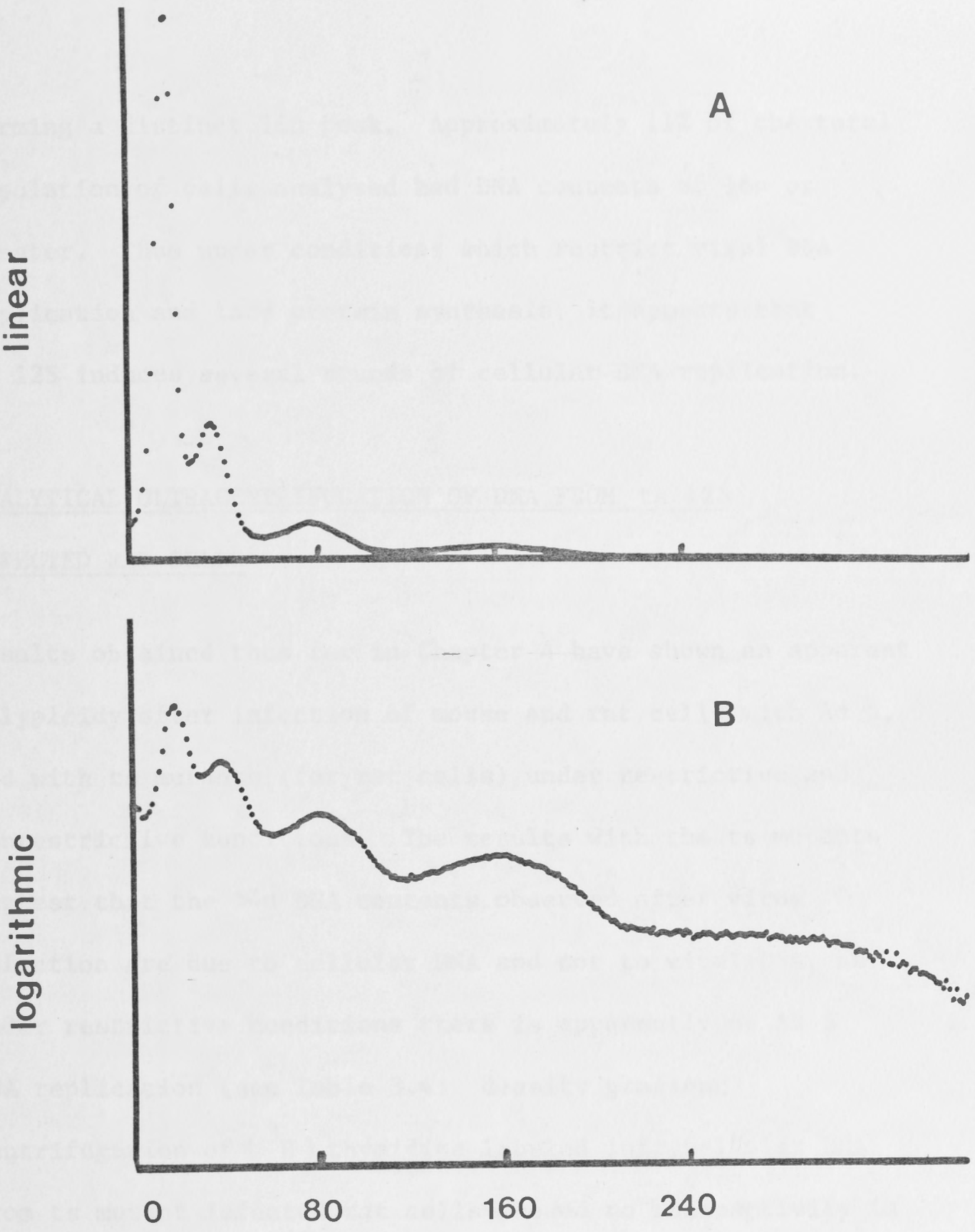
Cycling rat cells were infected with ts 125 and incubated at 39.5^{o}C and then cells were harvested and analysed by flow cytometry 3 and 6 days after infection. In this experiment, approximately 10^{6} cells were analysed and the results obtained 6 days after infection are shown in Fig. 4.11 as linear and semilogarithmic plots. 26% of cells were found to have DNA contents of 8n or greater, as well as a population of cells

FIGURE 4.11

Induction of cells with an octaploid DNA content by ts 125 at 39.5°C. Rat cells were infected with ts 125 and then incubated at 39.5°C for 6 days. At this time, cells were harvested and stained for flow cytometry. In this experiment approximately 10^6 cells were analysed. For more details of experiment refer to the text and for staining procedure see Section 4.2.2. This experiment was done using the FACS IV in Canberra.

(A) Linear plot of DNA histograms from ts 125 infected rat cells; (B) The same as in (A) except that the data is represented as a semilogarithmic plot.

In this experiment the G_1 peak position was set at about channel 20 in order to be able to see cells with >16n DNA contents on the visual display.


relative cell number
linear
A
logarithmic
B
0
80
160
240
channel number

forming a distinct 16n peak. Approximately 11% of the total population of cells analysed had DNA contents of 16n or greater. Thus under conditions which restrict viral DNA replication and late protein synthesis, it appears that ts 125 induces several rounds of cellular DNA replication.

4.3.3.4 ANALYTICAL ULTRACENTRIFUGATION OF DNA FROM ts 125 INFECTED RAT CELLS

Results obtained thus far in Chapter 4 have shown an apparent polyploidy after infection of mouse and rat cells with Ad 5, and with ts mutants (for rat cells) under restrictive and nonrestrictive conditions. The results with the ts mutants suggest that the >4n DNA contents observed after virus infection are due to cellular DNA and not to viral DNA, as under restrictive conditions there is apparently no Ad 5 DNA replication (see Table 3.4: density gradient centrifugation of [^{3}H] thymidine labeled intracellular DNA from ts mutant infected rat cells showed no radioactivity in the viral density position). In addition, even when Ad 5 DNA replication is occurring results presented in Section 3.3.8 suggest that it is unlikely that all of the observed increase in cell DNA content can be explained by viral DNA replication. However, in order to properly eliminate this as a possible explanation for the apparent induction by adenovirus of cells with >4n DNA contents, analytical ultracentrifugation of ts 125 infected rat cell DNA was done after incubation at permissive and nonpermissive temperatures.

FIGURE 4.12

Analytical ultracentrifugation of total intracellular DNA from rat cells infected with ts 125. Rat cells were infected at 36.5°C with ts 125, or mock infected, then incubated for 3 days at 39.5°C (the nonpermissive temperature for mutant replication), or 4 days at 32.5°C (permissive temperature). DNA was extracted with phenol as described in Sections 3.2.2 and 3.2.3, then centrifuged in CsCl (p = 1.707) to equilibrium (44,770 rpm for 2 days at 25°C). DNA was centrifuged both with and without Micrococcus lysodiekticus DNA standard (p = 1.731), and the results with standard are shown in this figure. UV absorbance at 265.5 nm was done on the centrifuged DNA and analysed by the method of Reisner (1980).

(a) MOCK infected, 32.5°C

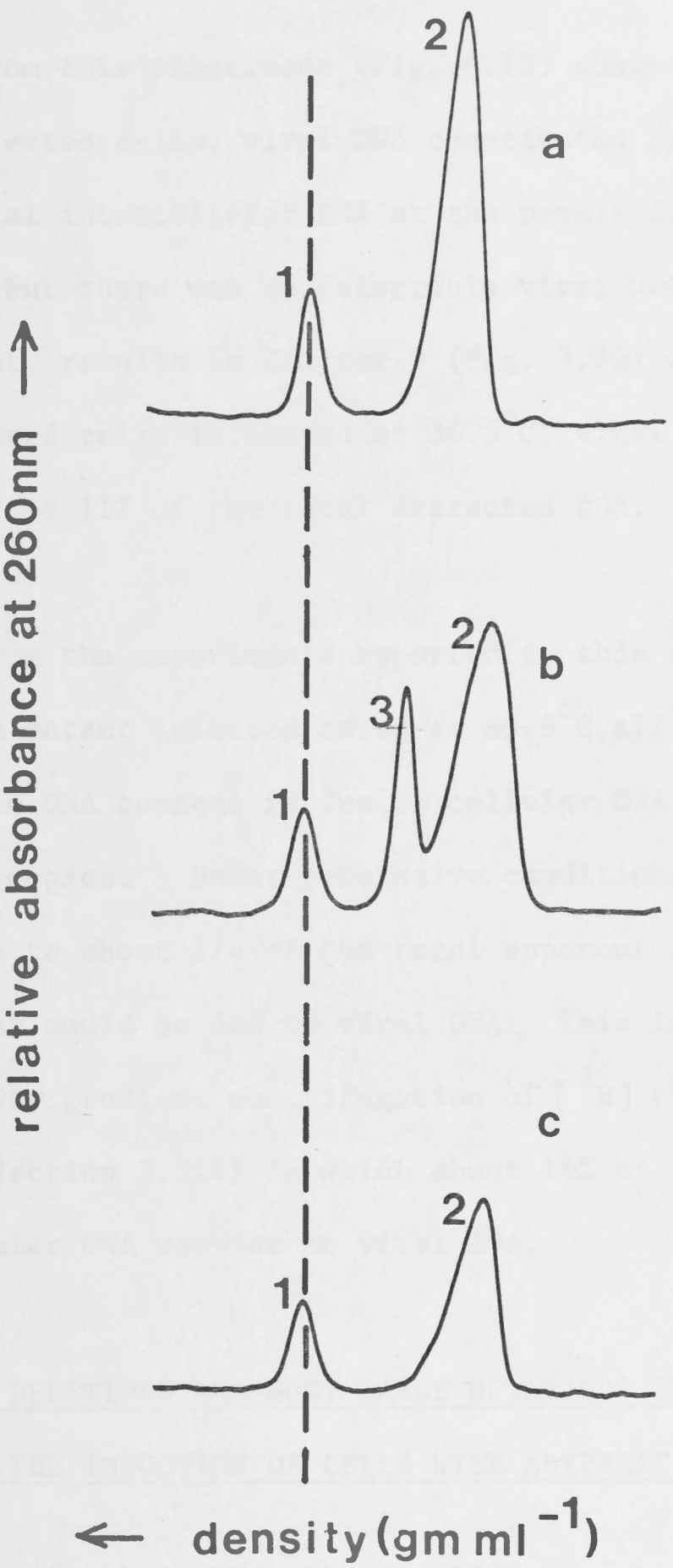
(b) ts 125 infected, 32.5°C

(c) ts 125 infected, 39.5°C

Labels: 1 - Micrococcus lysodiekticus DNA

2 - Rat cell DNA

3 - Ad 5 DNA


relative absorbance at 260nm →
a
1
2
b
1
3
2
c
1
2
← density (gm ml⁻¹)

Results from this experiment (Fig. 4.12) showed that in ts 125 infected cells, viral DNA constituted approximately 23% of total intracellular DNA at the permissive temperature (32.5°C), but there was no detectable viral DNA at 39.5°C. In contrast, results in Chapter 3 (Fig. 3.10) with wild-type Ad 5 infected cells incubated at 36.5°C, viral DNA constituted approximately 11% of the total extracted DNA.

Results from the experiments reported in this section show that in ts mutant infected cells at 39.5°C all of the increase in DNA content is due to cellular DNA, and not to viral DNA copies. Under permissive conditions at 32.5°C, up to about 1/4 of the total apparent increase in DNA content could be due to viral DNA. This is in agreement with density gradient centrifugation of [^{3}H] thymidine labeled DNA (see Section 3.3.4) in which about 16% of the total intracellular DNA was due to viral DNA.

4.3.3.5 EFFECT OF DELETIONS AND HOST-RANGE MUTATIONS IN THE Ad 5 GENOME ON THE INDUCTION OF CELLS WITH ABERRANT DNA CONTENTS

In order to further study the specific Ad 5 gene requirements for the aberrant cell cycle progression and DNA content of infected cells, mutants of Ad 5 in early regions 1A and 1B were studied. Mutant d1 312 is deleted in early region 1A and d1 313 and hr 7 are mutated in early region 1B (see Sections 3.1 and 4.1.3). d1 312 is totally defective for

mRNA synthesis from early region 1A (Lewis et al., 1979). In addition, mutant d1 313, whilst it is defective for some early region 1B functions, and behaves phenotypically like an E1B mutant, also affects early region 1A expression, presumably because it is deleted in the C-terminal region of 1A. The 4 major polypeptides from early region 1A detected by cell free translation are absent in d1 313 infected cells, and are replaced by 40 K and 36 K polypeptides (Lewis et al., 1979). hr 7 on the other hand synthesises wild-type levels of all mRNAs from early regions excepting 1B (Berk et al., 1979).

As for previous experiments reported in this chapter, the effect of the above mutants on rat cell cycle controls was investigated using flow cytometry. Results of the first experiments carried out at multiplicities of infection of 5.0 iu/cell and 20.0 iu/cell (Fig. 4.13) showed that mutants hr 7 and d1 313 both caused cell cycle alterations. Mutant hr 7 induced marked cell cycle changes such that at 20 iu/cell only 19.9% of cells remained in G_1 and 24.2% had DNA contents which were $>G_2$ diploid (see Fig. 4.13(C) and Table 4.3). Mutant d1 313 by contrast, whilst causing a decline in G_1 cells and an increase in S and G_2 cells, did not cause a dramatic increase in cells with $>G_2$ diploid DNA contents (see Fig. 4.13(G) and (H) and Table 4.3). d1 312 appeared to be totally negative under the same conditions having a DNA histogram which was almost identical to uninfected rat cell controls (see Fig. 4.13(E), (F) and Table 4.3).

FIGURE 4.13

DNA histograms of rat cells infected with deletion and host-range mutants of Ad 5. Cells were cultured and infected with mutants hr 7, d1 312 and d1 313 as previously described. 3 days after infection cells were harvested and stained for DNA content analysis by flow cytometry. For details of staining procedure refer to Section 4.2.2. These experiments were done using the FACS IV in Canberra.

(A) MOCK infected (experiment 1)

(B) hr 7, 5.0 iu/cell

(C) hr 7, 20.0 iu/cell

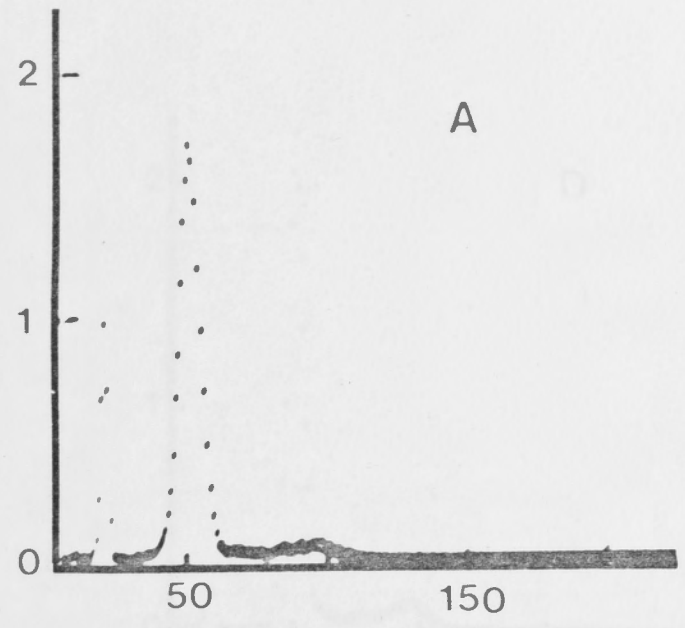
(D) MOCK infected (experiment 2)

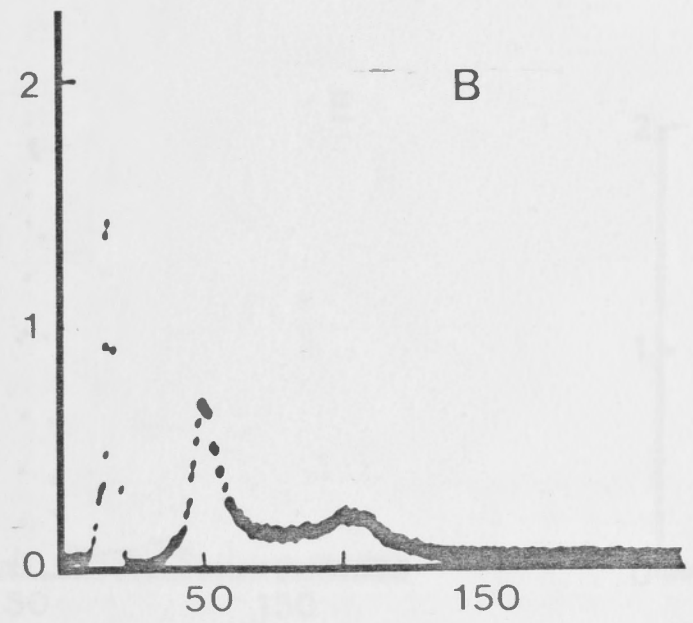
(E) d1 312, 5.0 iu/cell

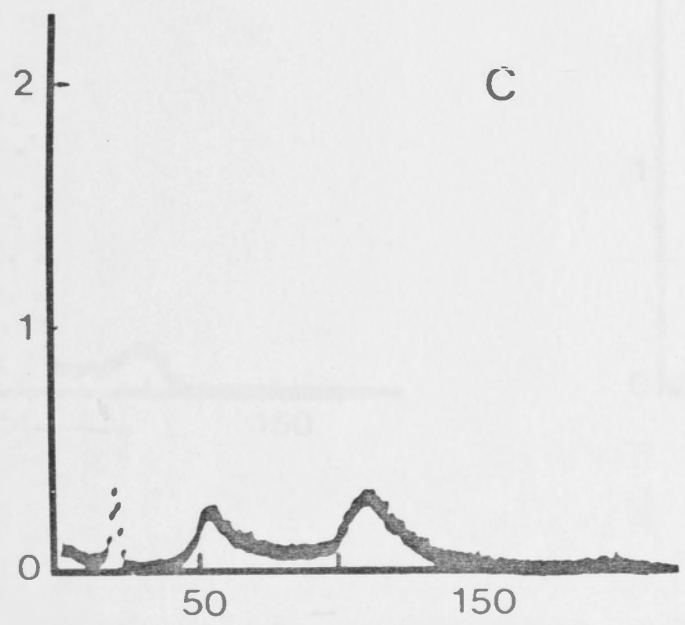
(F) d1 312, 20.0 iu/cell

(G) d1 313, 5.0 iu/cell

(H) d1 313, 20.0 iu/cell

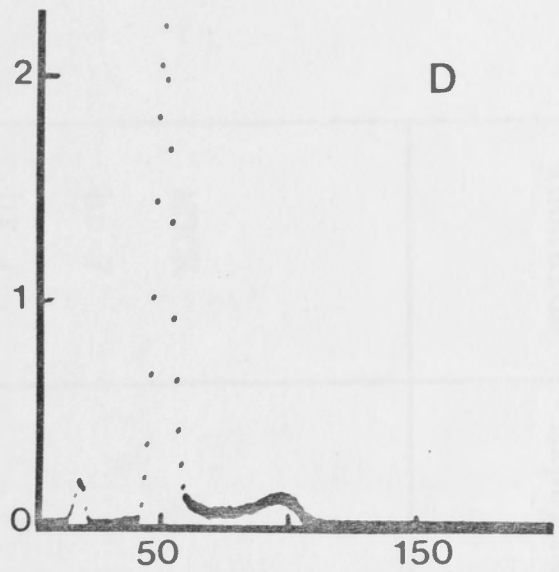






cell number (x10^{-3})

channel number


D
2
1
0
50
150

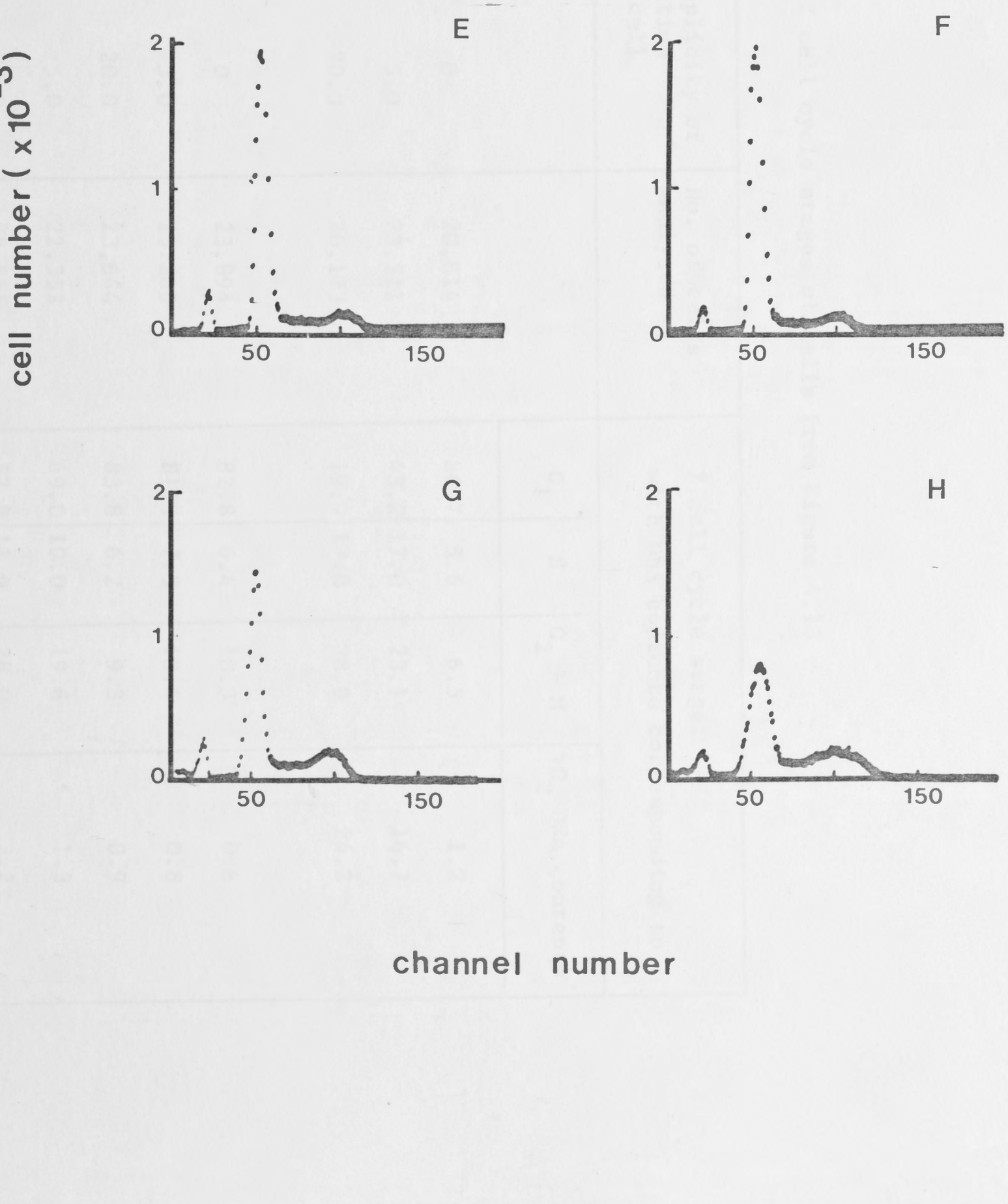

cell number (x10^{-3})
E
F
G
H
2
1
0
50
150
channel number

TABLE 4.3

Approximate % cell cycle stages of cells from Figure 4.13

Inoculum	Experiment	Multiplicity of Infection iu/cell	No. of cells analysed	% cell cycle stage with DNA contents corresponding to			
				G_1	S	G_2 + M	>G_2 DNA contents
MOCK	1	0	20,814	86.7	5.6	6.5	1.2
hr 7	"	5.0	23,511	43.2	17.0	23.1	16.7
hr 7	"	20.0	20,173	19.9	17.0	38.9	24.2
MOCK	2	0	23,894	82.8	6.4	10.3	0.6
dl 312	"	5.0	23.280	81.5	7.3	10.4	0.8
"	"	20.0	23,644	83.8	6.2	9.3	0.9
dl 313	"	5.0	22,352	69.0	10.0	19.6	1.3
"	"	20.0	22,555	57.8	11.9	28.0	2.2

These results seemed surprising mainly because of differences in potency between the mutants dl 313 and hr 7. hr 7 was clearly positive for induction of cell cycle abnormalities, whereas dl 313 gave a rather equivocal response.

In order to compare the cell cycle effects of these mutants with Ad 5 wild-type, and with each other in more detail, a series of multiplicities of infection from 1 to 16 iu/cell was studied for all 3 mutants and Ad 5 wild-type in the one experiment. Results of this experiment (Fig. 4.14) showed that Ad 5 wild-type and hr 7 all produced pronounced cell cycle alterations relative to an uninfected control which were multiplicity dependent and all resulted in a marked accumulation of cells with $>G_2$ diploid DNA contents. The multiplicity dependence of these two viruses was virtually identical (compare Fig. 4.14 (B-G) and (H-M). Over the same multiplicity range dl 313 caused some cell cycle alterations but this mutant proved to be much less potent than hr 7or Ad 5 (see Fig. 4.14 (T-Y), and dl 312 was totally defective (Fig. 4.14 (N-S). The results of a multiplicity of infection of 4.0 iu/cell were tabulated (Table 4.4) from this figure, and like the DNA histograms indicated marked cell cycle alterations by Ad 5 and hr 7; a lesser effect by dl 313; and no effect by dl 312.

Taken together, these results suggest that the adenovirus induced cell cycle alterations observed in these experiments is controlled by some gene product coded for by early region 1A; but with the reservations discussed in Section 3.4. In

FIGURE 4.14

Effect of varying the multiplicity of infection on cell cycle alterations by infection with Ad 5 wild-type and mutants hr 7, dl 312, and dl 313. Growing rat cells were infected with Ad 5 or mutants then incubated for 2 days at 36.5^{o}C. At this time cells were harvested and stained for DNA analysis by flow cytometry. For details of staining procedure refer to Section 4.2.2. This experiment was done using the FACS IV in Canberra.

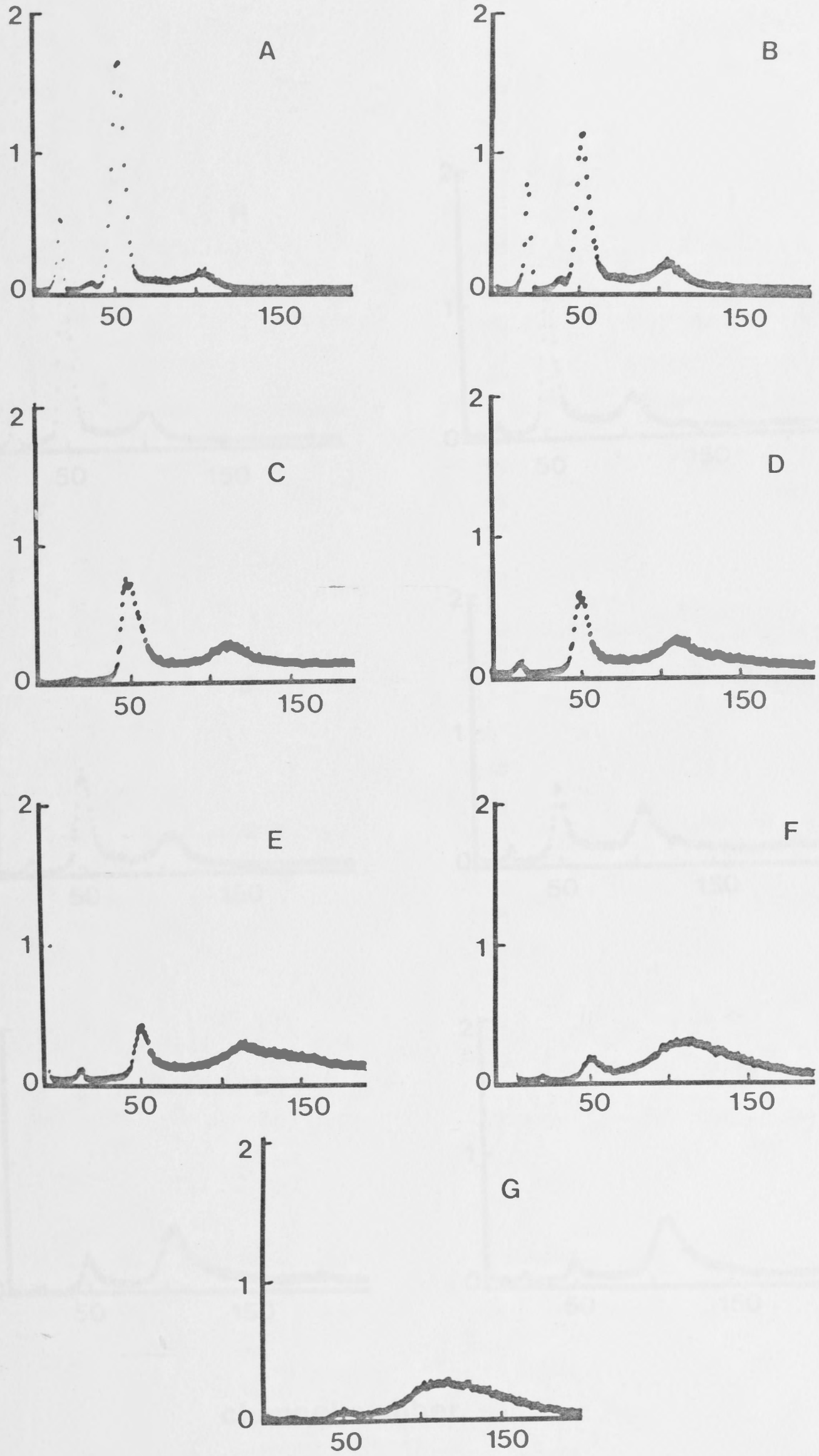
(A) MOCK infected

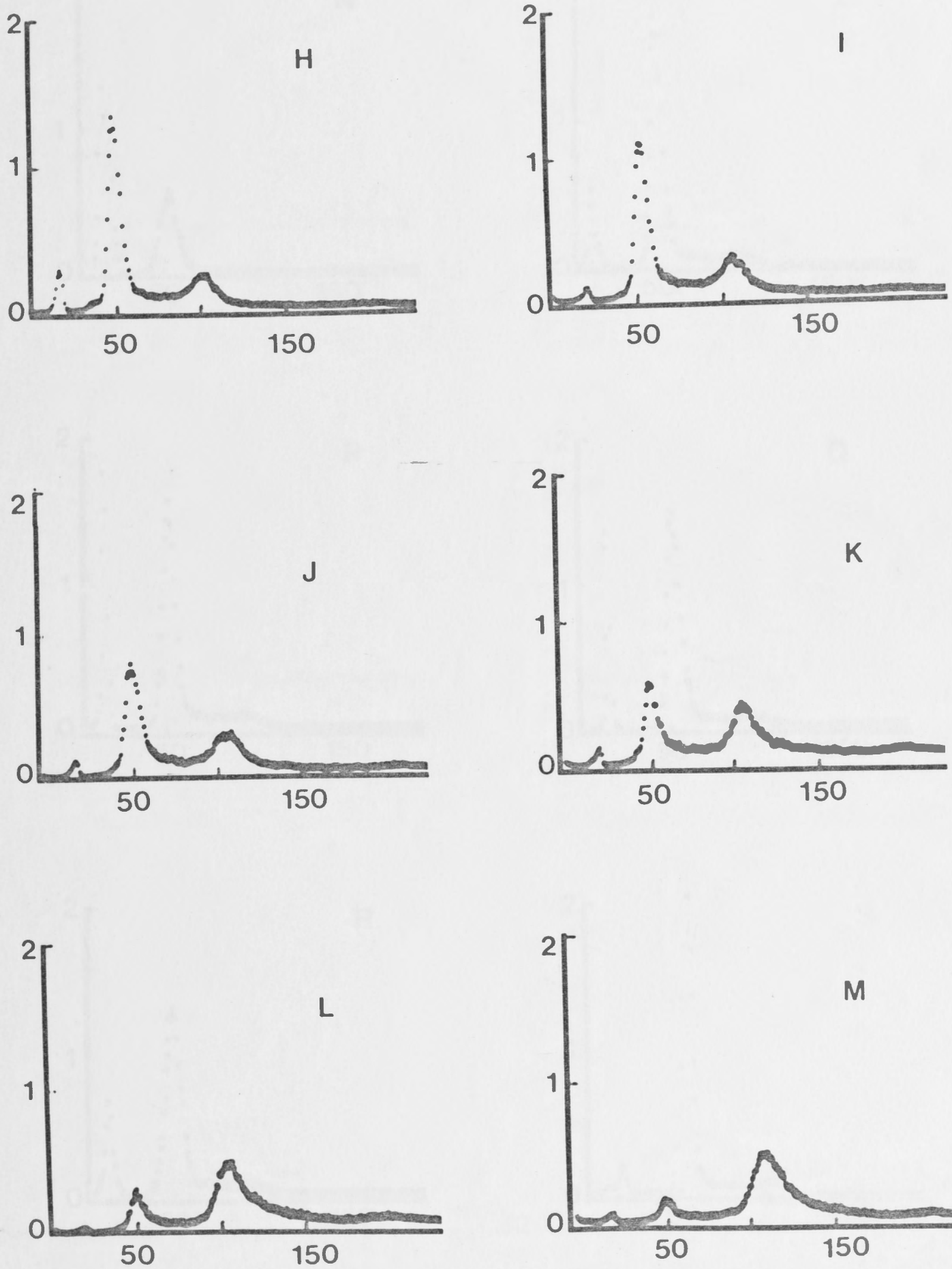
(B)-(G) Ad 5 wild-type: 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 iu/cell

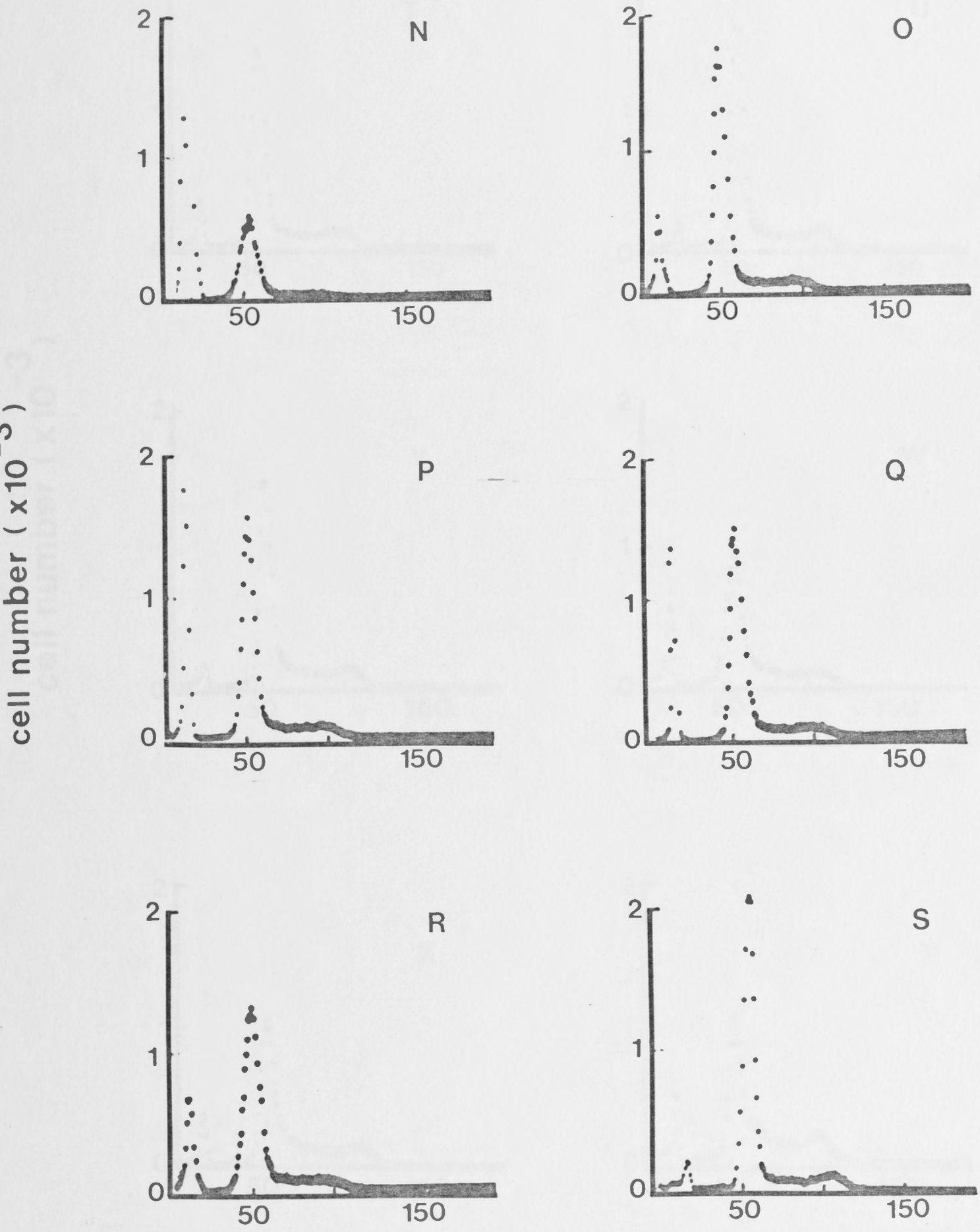
(H)-(M) hr 7: 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 iu/cell

(N)-(S) dl 312: 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 iu/cell

(T)-(Y) dl 313: 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 iu/cell


A
B
C
D
E
F
G
2
1
0
50
150
cell number (x 10 -3)
channel number


H
I
J
K
L
M
2
1
0
50
150
cell number (x10^{-3})
channel number


N
O
P
Q
R
S
2
1
0
50
150
cell number (x10^{-3})
channel number

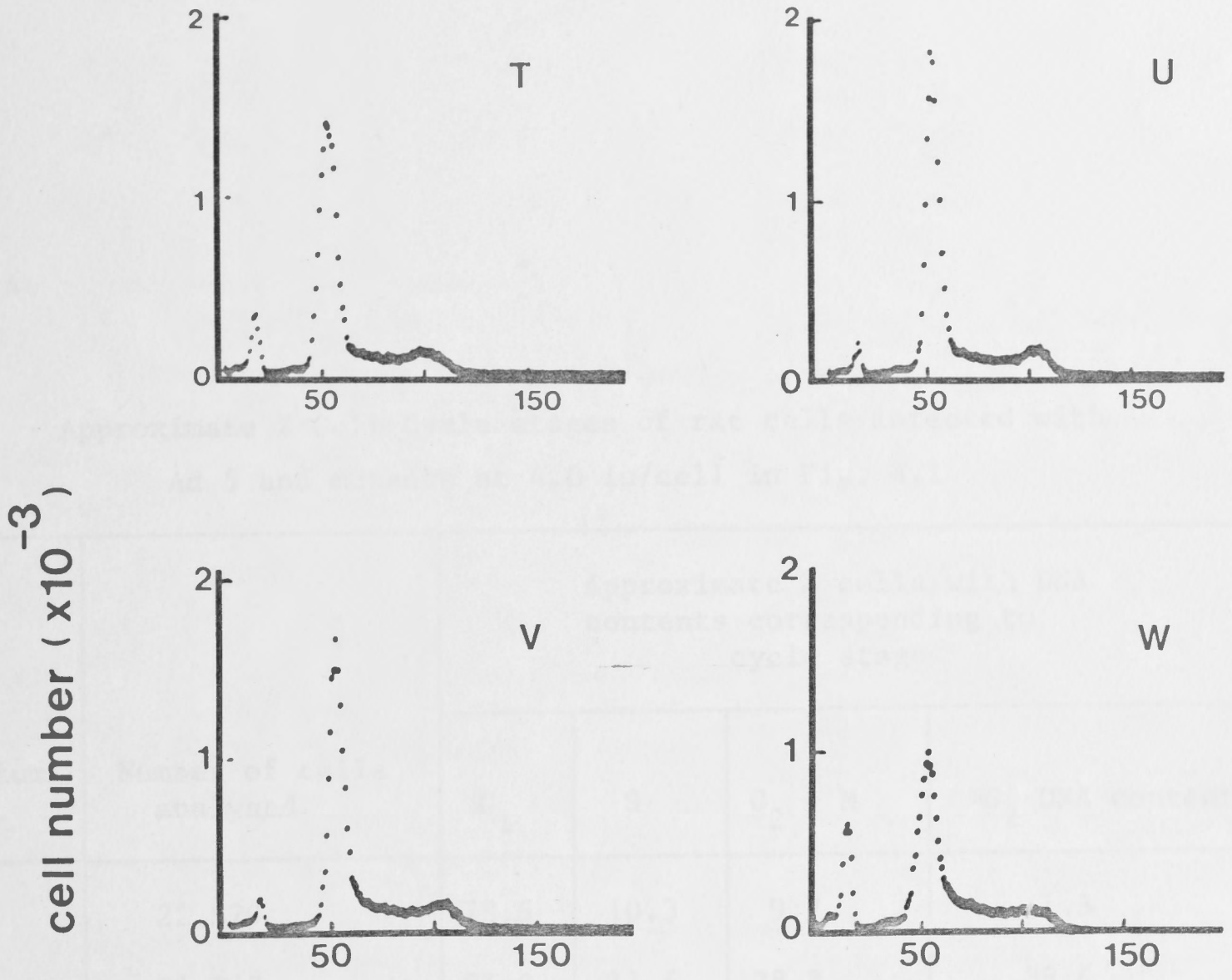

T
U
V
W
X
Y
2
1
0
50
150
cell number (x10^{-3})
channel number

TABLE 4.4

Approximate % Cell Cycle stages of rat cells infected with Ad 5 and mutants at 4.0 iu/cell in Fig. 4.14

Inoculum	Number of cells analysed	Approximate % cells with DNA contents corresponding to — cycle stage			
		G_1	S	G_2 + M	$>G_2$ DNA content
MOCK	22,529	78.5	10.3	9.7	1.5
Ad 5	24,017	21.0	11.6	28.8	38.6
hr 7	23,898	32.5	12.1	31.2	24.2
d1 313	21,769	60.9	20.5	14.8	3.8
d1 312	21,272	79.4	10.6	8.9	1.1

addition, preliminary results indicated that two other early region 1A defective mutants (hr 1 and hr 3; Harrison et al., 1977) were also negative for cell cycle alterations, providing further evidence for the above conclusion. This conclusion is discussed in more detail in Section 4.4.

4.4 DISCUSSION

Results of experiments reported in this chapter confirm and extend previous observations that adenovirus induces marked alterations to the normal rodent cell growth cycle (see Section 3.1; results in Chapter 3). These alterations result in a decline in the proportion of cells in the G_1 phase of the cell cycle relative to uninfected cells, and an increase in S and G_2 cells, as well as an accumulation of cells with $>G_2$ diploid (4n) DNA contents. Antiserum treatment and UV irradiation of the adenovirus particles prevent these cell cycle alteration suggesting that they require the expression of one or more virus gene products.

Experiments with the two ts mutants (ts 36 and ts 125) extended these observations and implied that the cell cycle alterations were dependent on the expression of one or more adenovirus early gene products, as the alterations occurred with both mutants at the nonpermissive temperature. These mutants are defective for synthesis of the Ad 5 DNA binding protein (ts 125) and the gene N product (ts 36) (see Section 3.1) implying that neither of these products is responsible for causing cell cycle alterations. These results argue against the early regions 2B (containing the ts 36 mutation) and 2A (containing the ts 125 mutation) being responsible, but with the caveats discussed in Section 3.4.

At the nonpermissive temperature, ts 125 induces some rat cells in the infected cultures to undergo several (>2) complete rounds

of DNA replication without completion of normal mitosis, so that 8n and 16n cells form. However, under permissive conditions for mutant replication, an abortive second round of cellular DNA replication occurs. Thus under nonpermissive conditions polyploids form whereas under permissive conditions most of the infected cells with $>G_2$ diploid DNA contents are aneuploid (i.e. 4n < DNA contents < 8n). This result under permissive conditions is the same as wild-type Ad 5 at all temperatures. This result suggests that some late virus gene product is synthesised which inhibits the cellular DNA replication induced by early gene functions. However, mutant ts 36 causes distinct polyploidy at both permissive and nonpermissive temperatures, although the cell cycle effects are much less pronounced than with either Ad 5 or ts 125. These differences are probably not due to differences in degrees of infection as determined by P antiserum staining of mutant infected cells in the same experiment. In several experiments in which the amount of viral DNA was estimated from density gradients (see Tables 3.4 and 3.5) the radioactivity appearing in the viral DNA density position in ts 36 infected cells at 32.5^{o}C was often lower than that observed for Ad 5 wild-type or ts 125 in the same experiment. This suggests that even at the permissive temperature this mutant shows delayed initiation of viral DNA replication implying that late viral antigen expression is concomitantly reduced. Quite why this is the case is unclear; however, it may be due to a second and as yet undetected mutation. Other unusual effects have also been reported with ts 36 (Ginsberg et al., 1974; Williams et al., 1974; van der Vliet and Sussenbach, 1975;

A.J.D. Bellett, B.F. Cheetham, and L.K. Waldron-Stevens, submitted for publication). Thus the explanation that the differences in cell cycle alterations between permissive and nonpermissive conditions is due to late viral gene expression still seems reasonable. Since ts 125 has a host-range defect in mouse cells (Younghusband et al., 1979), and in these cells distinct tetraploids occur at the permissive temperature (see Fig. 4.4), the above explanation can also account for this observation.

Analytical ultracentrifugation and integrated computer analysis of Ad 5 and ts 125 infected rat cells at permissive and nonpermissive temperatures, showed clearly that there is insufficient viral DNA to account for the aberrant DNA contents observed by flow cytometry. At 39.5^{o}C in ts 125 infected cells, no viral DNA was detected at all by this analysis implying that all the aberrant DNA contents caused by virus infection are due to changes in cellular DNA content. The adenovirus infected cells with DNA contents >G_2 diploid (4n) are therefore likely to be aneuploid and polyploid cells, although without counting the chromosomes of these cells this cannot be formally concluded. It does, however, seem a reasonable conclusion as polyploidy was clearly established in SV 40 infected cells (Lehman, 1974), in Ad 12 infected cells (McDougall et al., 1974) and Ad 5 infected mouse cells frequently contain >40 chromosomes (Murray et al., 1982).

Experiments with dl and hr mutants provided evidence that the adenovirus transcriptional unit responsible for causing rodent cell cycle alterations and polyploidy is early region 1A. Both mutants in early region 1B (hr 7 and dl 313) cause cell cycle alterations, although dl 313 is less potent than Ad 5 or hr 7. This region is therefore not responsible for causing polypolidy; the mutant in early region 1A, dl 312, was negative, suggesting that expression of some protein from early region 1A was responsible for causing the observed cell cycle alterations.

If the negative result obtained with hr 1 (see Section 4.3.3.5) is correct (A. Bellett, personal communication), then the number of possible protein candidates for inducing cell cycle alterations is reduced, as hr 1 synthesises only 42 K and 54 K polypeptides from early region 1A compared to Ad 5 wild-type, which synthesises 42 K, 48 K, 52 K and 54 K polypeptides (Lewis et al., 1979). Alternatively, Ricciardi et al., (1981) reported only 2 polypeptides synthesised from early region 1A in a wild-type infection, only one of which (a 48 K polypeptide) was synthesised after hr 1 infection. These observations suggest that the polypeptides responsible for causing cell cycle alterations are one or both of the 48 K and 52 K polypeptides detected by Lewis et al., (1979) or the 48 K polypeptide detected by Ricciardi et al., (1981).

Other evidence for the conclusion that early region 1A gene products are responsible for causing cell cycle alteration in Ad 5 infected cells comes from the lowered potency of dl 313. This mutant is not only partially defective for synthesis of early region 1B products, but also has modified early region 1A expression (see Section 4.3.3.5). All "normal" in vitro translated early region 1A polypeptides are missing in this mutant and are replaced by 36 K and 40 K polypeptides instead (Lewis et al., 1979). Presumably these polypeptides can still bind to the cellular substrate required for initiating a cell cycle but not with the same efficiency (specificity) as the polypeptides normally synthesised from this region. Thus for a given multiplicity of infection dl 313 would be comparatively less effective at inducing polyploids.

As was discussed in Section 3.4 however, the conclusion that early region 1A is responsible for inducing cell cycle alternations must still be considered indefinite until early region 4 is excluded as a candidate. A possible experimental approach to test this is discussed in Section 7.6.

The hr and dl mutants discussed above are defective for transformation of rat embryo fibroblasts and rat embryo brain cells (Graham et al., 1978; Shenk et al., 1979), but complementation of the mutants does cause transformation (Graham et al., 1978). However, experiments reported in this chapter showed that two of these rodent cell transformation defective mutants, hr 7 and dl 313, induced cell cycle

alterations and polyploidy in rodent cells. This suggests therefore that the polypeptides required to initiate a cell cycle and polyploidy are not the only polypeptides required to initiate transformation. That is, at least one of the other early region 1A polypeptides (42 K and 54 K polypeptides of Lewis et al., (1979) or the 51 K polypeptide of Ricciardi et al., (1981)) as well as one or more gene products from early region 1B may be the minimum requirements for transformation of rodent cells, as a fragment of DNA containing only early regions 1A and 1B is sufficient to transform cells (see Sections 3.1 and 3.4). However, other observations suggest that the regulation of transformation is not as simple as these results imply.

The frequency of adenovirus induced cellular transformation is altered by mutations in two other transcriptional units: in the region coding for the DNA binding protein (ts 125) (E2A) and in the region coding for the gene N product (e.g. ts 36 and ts 37) (E2B) (see Section 3.1). In addition, mutants in early region 1A (such as d1 312 and hr 1) will cause abortive transformation of baby rat kidney cells (Graham et al., 1978) and incomplete transformation of rat cells occurs with d1 313 (Shiroki et al., 1981). These abortive and incomplete transformants are phenotypically transformed, but Graham et al., (1978) were unable to establish immortal cell lines from these region 1A transformed foci or grow them in soft agar (d1 313 transformants; Shiroki et al., (1981)). In this respect these abortive transformants are unlike those described by

Stoker (1968) for polyoma virus in which after several generations the transformed cells reverted to a normal cell phenotype. The abortive transformants isolated after infection with adenovirus early region 1A or 1B mutants may occur as a result of some cellular protein partially complementing the virus defects and therefore leading to semitransformed cells. Such an explanation has been used to account for the "multiplicity dependent leakiness" of the early region dl mutants (Shenk et al., 1979). These additional observations suggest that there are other factors (both viral and cellular) that play a role in the regulation of transformation.

Taking together the above observations and the experiments reported in this chapter, it is proposed that adenovirus induces transformation of cells by first inducing an aberrant cell cycle by expression of one or more early region 1A polypeptides (Lewis et al., 1979 ; Ricciardi et al., 1981) from a fragment of DNA containing early region 1A which is likely to be integrated into the host genome (see Section 3.1 and 3.4). Once this first initiation event has taken place these early region 1A polypeptides, in conjunction with early region 1B products, contribute to this abnormal phenotype resulting in cellular transformants. Structural chromosome aberrations and polyploidy followed by abnormal segregation would introduce genetic variability into the cell population. The frequency with which these events occur is probably also dependent on the expression of some cellular genes and other

viral genes such as early regions 2A and 2B. Also, continued expression of cellular genes may or may not be dependent on continued expression of viral genes and integration of viral DNA segments. This requirement would depend on the cell type, the kind of assay in which the transformants were selected, and the subsequent conditions in which the transformants were grown. This kind of model would account for why in some transformed cells some adenovirus specific proteins are consistently detected (e.g. Lassam et al., 1979); why in other cases no one virus specific protein is consistently detected (e.g. Green et al., 1979); why some adenovirus-induced tumors lack T antigen (Stenback et al., 1973; Asch et al., 1979), and have a deletion of the transformation genes (May et al., 1978), and why in some transformants no adenovirus specific DNA or proteins are detected at all (e.g. Bellett and Younghusband, 1979; Paraskeva and Gallimore, 1980).

CHAPTER 5

REGULATION OF CELLULAR DNA REPLICATION BY CYCLIC AMP AND CALCIUM IN NORMAL AND Ad 5 INFECTED RODENT CELLS

5.1 INTRODUCTION

5.1.1 CYCLIC NUCLEOTIDES AND CELLULAR PROLIFERATION

Ever since the early observations of Bürk (1968) that cell proliferation could be prevented with exogenous cyclic AMP (cAMP) or theophylline (an inhibitor of the phosphodiesterase enzymes, one of which degrades cAMP), and that the level of the enzyme that synthesises cAMP (adenylate cyclase) was lower in polyoma virus transformed cells, many groups have tried to demonstrate a major regulatory role for cAMP in cell proliferation. A general hypothesis was developed over the years that low levels of cAMP, and high levels of cyclic 3', 5'-guanosine monophosphate (cGMP) are correlated with cell proliferation (the Yin Yang hypothesis; see Goldberg et al., 1974). Conversely, high cAMP and low cGMP correlated with a nonproliferative (G_1-arrested) state. Although many studies showed that cAMP or dibutyryl cyclic AMP (dbcAMP; a membrane permeable analogue of cAMP) inhibited cell division, and in transformed cells cAMP levels were lower than in the parent strain (see reviews by Pastan and Johnson, 1974; Ryan and Heidrick, 1974), other studies showed that cAMP or agents which increase the intracellular cAMP concentration stimulated cell division (MacManus and Whitfield, 1969; see review by Whitfield et al., 1973a) or had no effect on cell division (Rebhun et al., 1973; Coffino et al.,1975 a,b). A similar set of

contradictory observations has been made for the role of cGMP in controlling cellular proliferation. In some cells cGMP levels have been shown to increase when cells are stimulated to divide (Hadden et al., 1972) and in other cases cGMP analogues can themselves stimulate cells to enter DNA synthesis (Seifert and Rudland, 1974; Weinstein et al., 1974). However, under some conditions cGMP inhibits cell division (Whitfield et al., 1973b).

The role of cyclic nucleotides in cellular proliferation has recently been reviewed extensively by Rebhun (1977) and to a lesser extent by Hunt and Martin (1980), and both reviews agree that the evidence for cyclic nucleotides as universal regulators of cellular proliferation is at best equivocal. In their conclusions Hunt and Martin (1980) summarise their feelings with the statement:

> "Variations in cyclic nucleotide levels during the cell cycle have been observed but there is little evidence to suggest that they are regulators of cell timing."

The studies discussed above, whilst not ruling out the possibility that cyclic nucleotides are important proliferative regulators in some cells, do tend to exclude them from being universal regulators of cellular proliferation. No single general hypothesis can therefore explain all of the observations concerning cyclic nucleotides and proliferation.

Cyclic AMP appears to exert its effects exclusively via interaction with protein kinases, which catalyse the addition of phosphate groups generally to threonine or serine residues in different

protein substrates (refer to Hunt and Martin, 1980; Weller, 1979). These phosphorylated proteins are often enzymes themselves implying that the effect of the initial phosphorylation can be "amplified", and therefore have an effect on many different cellular functions. The best characterised example of the way cAMP exert its "amplifier" effects is in the activation of a cascade of phosphorylation events which regulate glycogen metabolism in liver (reviewed in Weller, 1979). Also, cAMP-dependent protein kinases appear to activate other enzymes including ornithine decarboxylase (ODC) (Klimpel et al., 1979). This enzyme is the first and rate-limiting enzyme in the biosynthetic pathway for polyamines, the synthesis of which is normally mandatory for entry into DNA synthesis (see for example, Fillingame et al., 1975; Klimpel et al., 1979; Cheetham and Bellett, 1982). The cyclic AMP-dependent protein kinases also affect cell membranes by altering microtubule arrangement (see review by Krebs and Bearo, 1979). Thus there are probably many ways in which cAMP could affect cellular proliferation. It is its role as an overall regulator of proliferation that is questionable.

On occasions however, the cAMP analogue dbcAMP has been profitably used to investigate some control mechanisms of mammalian cell proliferation. To cite one example, Pardee (1974) was able to show that serum starvation, amino acid deprivation, and treatment with dbcAMP all prevented cell cycle progression in BHK 21 cells at approximately the same point in the cell cycle (in G_1). Such experiments led to the proposal of a single G_1 regulatory block

at which cells accumulated if growth conditions were not favorable. This has been termed the "restriction point" (Pardee, 1974).

In this chapter dbcAMP is used as a tool to manipulate cellular proliferation as Pardee used the molecule, but not with the intent of claiming it as a universal or primary regulator of cell division.

5.1.2 CONTROL OF CELLULAR PROLIFERATION WITH CATIONS

In the last ten years evidence has been accumulating which suggests that monovalent and divalent cations play a major role in controlling cellular proliferation in vitro; in this context Ca^{2+}, K^{+}, and Na^{+} have featured most prominently. Much of the work in the early and mid-seventies concerning Ca^{2+} regulation of proliferation has been reviewed by Berridge (1975) and Rebhun (1977), but recently some careful and interesting studies have been published concerning the regulatory controls of Ca^{2+} and other cations. Some of these are discussed below.

Frantz et al.(1980) studied the effect of inhibiting the cell membrane (Na^{+} - K^{+}) pump on the cell growth cycle using balb/c-3T3 cells. Treatment of cells with ouabain, an agent which blocks the (Na^{+} - K^{+}) pump, reduced the intracellular K^{+} concentration. When this concentration was reduced below a critical threshold (50 to 60mM) protein synthesis declined and entry into DNA synthesis (S phase) was prevented. This

inhibition of K^+ influx did not affect the induction of competence by platelet-derived growth factor (see Chapter 1), but did prevent the plasma mediated progression into S phase. Thus K^+ ions are required in order for the initiation events to occur, and it would appear that these ions probably exert their effects by preventing synthesis of one or more proteins required for entry into DNA synthesis. A similar requirement for K^+ in cell cycle progression has recently been reported for concanavalin A stimulated human lymphocytes (Szamel et al., 1980) and cultured neuroblastoma cells (Mummery et al., 1981).

By contrast, the requirements for critical K^+ and Na^+ concentrations for normal cell cycling appear to be relaxed in some cells. Lubin (1980) examined the effects of ouabain inhibition of the (Na^+ - K^+) pump on the proliferation of normal 3T3 cells and SV 40 transformed 3T3 cells (called ATCC SV-T2). A decrease in intracellular K^+ concentration from 165mM to 120mM reduced the rate of normal cell proliferation, whereas a decline in K^+ concentration to 50mM had no effect on the proliferation rate of SV 40 transformed cells.

An interesting Ca^{2+} requirement has been reported for cultured mouse mastocytoma cells. Knightbridge and Ralph (1981) showed that treatment of these mouse cells with dbcAMP and theophylline, or serum starvation, caused them to accumulate in the G_1 phase of the cell cycle but the cAMP arrest point was 1.5 to 2 h after the low serum arrest point. In order for cell cycle progression

to continue after removal of the blocking agents (or serum addition) protein synthesis was required and Ca^{2+} above a critical threshold concentration. Ca^{2+} ions were required to be above this threshold for a period of only 5 minutes after the blocking agent was removed, after which time these cells became insensitive to lowering the Ca^{2+} concentration. Thus in these cells a single pulse of Ca^{2+} is required in order that cells might progress through G_1 to S phase.

A comparison of divalent cation (Ca^{2+}, Mg^{2+}) requirements for normal and transformed human lung fibroblasts has been made (McKeehan and McKeehan, 1980). Both Ca^{2+} and Mg^{2+} appeared to be essential for cell cycling to continue in normal lung fibroblasts but this requirement was considerably reduced after they had been transformed with SV 40. This reduced sensitivity to cation regulation was particularly marked for Mg^{2+}.

Studies such as those mentioned above emphasise the importance of critical threshold concentrations of several different cations for maintaining cells in a proliferating state. However, as for cyclic nucleotides, cations do not always regulate cell cycle progression in the same way. For example, Hennings et al., (1980) showed that raising the culture medium Ca^{2+} concentration to the usual 1.2mM totally inhibited DNA replication of cultured mouse epidermal cells. At the normal Ca^{2+} concentration these cells became stratified and eventually terminally differentiated. A different result was also obtained by Damulji and Riley (1979)

who showed that a transient reduction in Ca^{2+} concentration was required just before the onset of replicative DNA synthesis in mouse 3T3 cells. Thus whilst Ca^{2+} (and other cations) may be important for maintenance of cell growth, the particular role for Ca^{2+} probably depends very much on the cells and their physiological responses to culture growth conditions. Ca^{2+} ions appear to mediate their action via interaction with a particular protein as is the case with cyclic AMP. This is the calcium dependent regulator protein now known as calmodulin. Ca^{2+} binds to calmodulin which in turn regulates the activity of a number of enzymes. For example, a Ca^{2+}-calmodulin complex has been shown to regulate the activities of a cyclic nucleotide phosphodiesterase, adenylate cyclase, and a protein kinase (see review by Wolff and Brostrom, 1979). Thus Ca^{2+} action and cyclic nucleotides would appear to be intimately interrelated. Ca^{2+} and cyclic AMP have been reported to have marked effects on cellular morphology. Many studies have shown that Ca^{2+} causes cells to have a flattened elongate shape. If culture medium is depleted of Ca^{2+}, cells become rounded and growth rate is reduced (Dulbecco and Elkington, 1975; see studies cited by Rebhun, 1977). This effect of Ca^{2+} seems to be primarily due to modulation of the microtubule assembly in the cell membrane (and in the mitotic spindle) by inhibiting tubulin polymerisation (Weisenberg, 1972; see review by Dedman et al., 1979). Ca^{2+} can therefore have a number of effects on cellular proliferation controls, probably affecting several different functions in a similar manner to the cyclic nucleotides.

5.1.3 ALTERED CONTROLS OF CELLULAR PROLIFERATION IN ADENOVIRUS INFECTED CELLS

The many observations that different serotypes of adenovirus induced at least one round of cellular DNA replication in noncycling cells (see Strohl, 1969; Laughlin and Strohl, 1976a, b; other references cited in Section 3.1; and experiments reported in Chapter 3) suggested that adenovirus acts in some direct way to modify the controls of cellular proliferation. In addition, the observations that adenovirus induced DNA synthesis in serum arrested cells (e.g. Fig. 3.2), confluent cells (e.g. Fig. 3.3), and in differentiated cells (Fig. 3.8) suggested further that the virus might be able to modify multiple control points of cellular proliferation.

Two observations concerning the response of Ad 12 and SV 40 induced cellular DNA replication to changes in cyclic AMP levels seemed interesting in this context. Zimmerman et al.,(1972) showed that concentrations of dbcAMP having only a small inhibitory effect (less than 40% compared to untreated controls) on serum induced DNA replication completely inhibited Ad 12 induced DNA replication in BHK 21 cells. Thus in this case, Ad 12 modified the controls of cellular proliferation which made cells more sensitive to cyclic AMP. By contrast, induction of cellular DNA replication by SV 40 in monkey cells (CV-1) was only slightly affected by theophylline concentrations (which inhibits cyclic AMP phosphodiesterase thus elevating the intracellular cAMP concentration) which totally inhibited DNA

synthesis in uninfected cells (Rundell and Cox, 1979). Therefore this virus also can modify controls of cellular proliferation other than just at the low serum arrest point (see Section 3.1).

Another observation which prompted the investigations reported in this chapter is that adenovirus transformants are generally isolated in culture medium containing only 0.1mM Ca^{2+} ions, which has been reported to give more reproducible quantitation of transformation frequency and allowed the development of transformed cell lines (Freeman et al., 1967). This result suggested that adenovirus might alter cell proliferation controls in some way which made cells insensitive to Ca^{2+} ion modulation and might be important in the selection of adenovirus induced transformants.

As well as these studies, in the last 3 years several findings have been published which imply that adenovirus can overcome a number of cellular regulatory points. Adenovirus was shown to overcome two separate arrest points in temperature sensitive hamster cell cycle mutants (Rossini et al., 1979 a, b) and to stimulate DNA replication in BHK cells without a concomitant increase in rRNA synthesis (Pochron et al., 1980).

With the above background, in this chapter I decided to modulate normal rodent cell proliferation with dbcAMP and by lowering the Ca^{2+} concentration, and determine what effect Ad 5 infection had on these cells. More detailed and different evidence

was provided for adenovirus induced cell cycle control alterations.

5.2 METHODS

In this chapter DNA synthesis was measured both by [^{3}H] thymidine incorporation into acid insoluble material (see Section 2.2.1) and by equilibrium gradient centrifugation (see Section 3.2.2). Total cell cycle analysis was done for one experiment using the technique of flow cytometry (Section 4.2.2). In addition, in one experiment the effect of serum and Ad 5 on cyclic nucleotide phosphodiesterase activities was assayed. The principle and details of this method are discussed in Section 5.3.4.1.

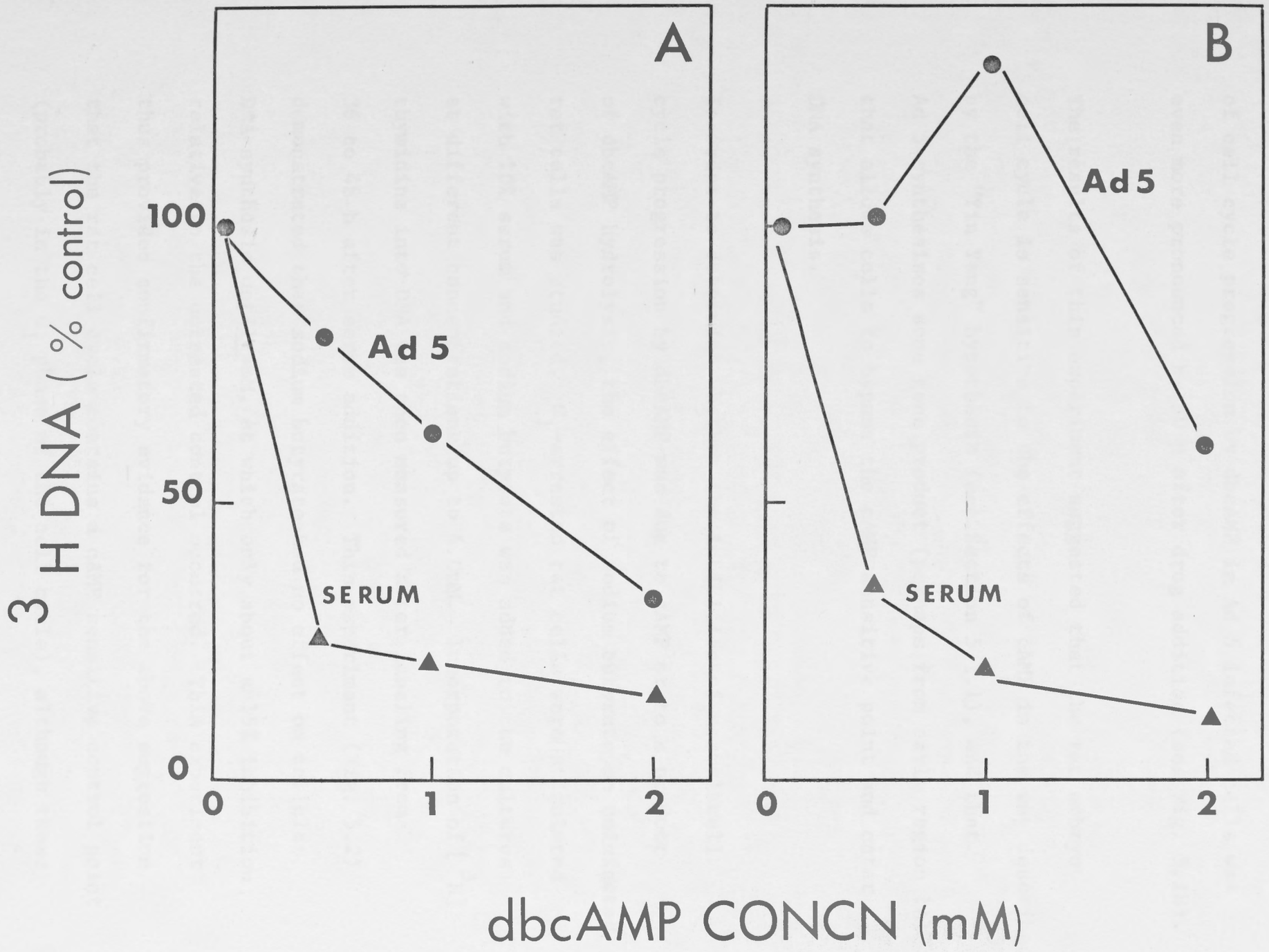
5.3 RESULTS

5.3.1 EFFECT OF DIBUTYRYL CYCLIC AMP ON THE INDUCTION OF DNA SYNTHESIS BY SERUM AND Ad 5 IN RAT CELLS

Observations by Frank (1972) that DNA synthesis was reduced in cultured embryonic rat fibroblasts (resulting in G_1-arrest) after treatment with 1.0mM dbcAMP, provided the starting point for the experiments described in this chapter. In the first experiment G_1-arrested rat cells were stimulated into a cell cycle by the addition of fresh serum growth factors or by Ad 5 infection. Some cultures were then treated with dbcAMP (at indicated concentrations) and then cells were labeled with [^{3}H] thymidine from 12 to 24 h and 48 to 60 h later. Results of this experiment (Fig. 5.1) showed that dbcAMP inhibited [^{3}H] thymidine incorporation into the DNA of serum treated cells substantially,

FIGURE 5.1

Effect of dbcAMP on induction of DNA synthesis in G_1-arrested rat cells infected with Ad 5 or treated with 10% FCS. Cells were infected or treated with serum, and dbcAMP was then added at the indicated concentrations. Cells were labeled with [^{3}H] thymidine from 12 to 24 h (A) or 48 to 60 h (B) after the addition of virus or serum, and DNA synthesis was measured as described in Section 2.2.1. Results are expressed as percentage radioactivity (c.p.m.) incorporated into untreated (no dbcAMP), serum treated and virus infected controls. Untreated control DNA synthesis represented 7.8- and 3.5-fold stimulations above mock infected cells for serum treated and virus infected cells for (A) and 4.6- and 2.6-fold stimulations for (B).


A
B
100
50
0
Ad 5
SERUM
Ad 5
SERUM
0
1
2
0
1
2
^{3}H DNA (% control)
dbcAMP CONCN (mM)

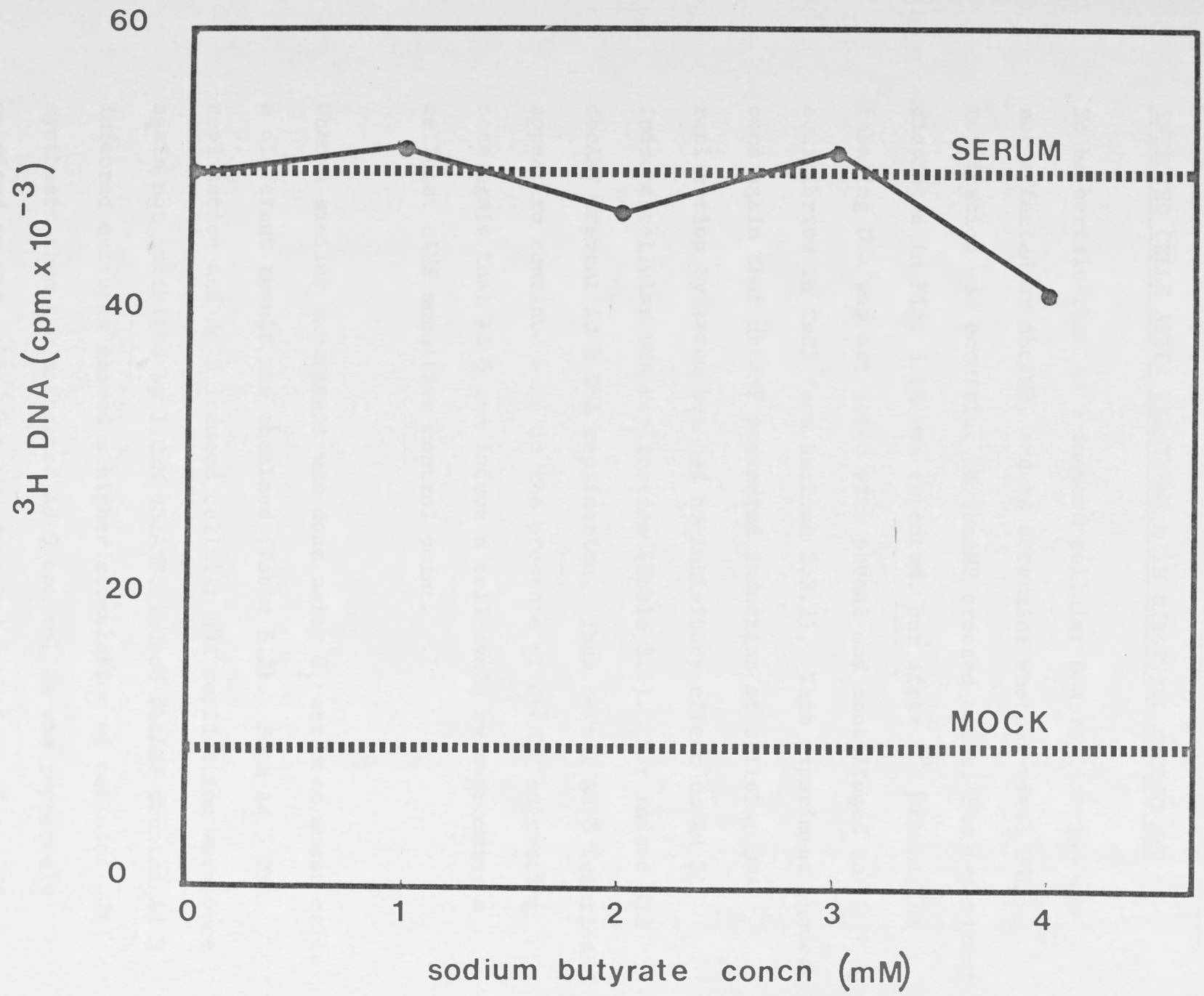
such that by 24 h after addition of dbcAMP incorporation was only about 20% of untreated control values. By contrast, incorporation was reduced only to about 65% of control values in Ad 5 infected cells. This relative resistance to inhibition of cell cycle progression by dbcAMP in Ad 5 infected cells was even more pronounced by 60 h after drug addition (see Fig. 5.1B).

The results of this experiment suggested that the rat embryo cell cycle is sensitive to the effects of cAMP in the way described by the "Yin Yang" hypothesis (see Section 5.1.1), and that Ad 5 synthesises some gene product (perhaps from early region 1A) that allows cells to bypass the cAMP sensitive point and enter DNA synthesis.

In order to determine whether the inhibition of normal cell cycle progression by dbcAMP was due to cAMP or to a product of dbcAMP hydrolysis, the effect of sodium butyrate on uninfected rat cells was studied. G_1-arrested rat cells were stimulated with 10% serum and sodium butyrate was added to the cultures at different concentrations up to 4.0mM. Incorporation of [^{3}H] thymidine into DNA was then measured after labeling from 36 to 48 h after serum addition. This experiment (Fig. 5.2) demonstrated that sodium butyrate had no effect on cellular DNA synthesis until 4mM, at which only about a 15% inhibition relative to the untreated control occurred. This experiment thus provided confirmatory evidence for the above suggestion that the rat cell cycle contains a cAMP sensitive control point (probably in the G_1 phase of the cell cycle), although these

FIGURE 5.2

DNA synthesis in serum stimulated rat cells treated with sodium butyrate. Quiescent rat cells were stimulated with 10% serum and then sodium butyrate was added to some cultures at indicated concentrations. Cells were labeled from 36 to 48 h with [^{3}H] thymidine and the incorporation of label into acid insoluble DNA was measured. The hatched lines represent an untreated G_1-arrested culture (MOCK) and a serum stimulated (SERUM) control.


60
40
20
0
^{3}H DNA (cpm x 10^{-3})
SERUM
MOCK
0
1
2
3
4
sodium butyrate concn (mM)

experiments do not exclude the possibility that 5' -AMP is the controlling agent.

5.3.2 CsCl DENSITY GRADIENT CENTRIFUGATION OF SERUM TREATED AND Ad 5 INFECTED CELLS AFTER TREATMENT WITH DIBUTYRYL CYCLIC AMP

To be certain that Ad 5 induced cellular DNA replication was not affected by dbcAMP, and to determine whether viral DNA replication was occurring in dbcAMP treated cells, the experiment displayed in Fig. 5.1B was repeated, but after [^{3}H] thymidine labeling DNA was extracted with phenol and centrifuged to equilibrium in CsCl (see Section 3.2.2). This experiment showed once again that dbcAMP prevented induction of cellular DNA replication by serum but had no inhibitory effect on Ad 5 induced cellular DNA replication (Table 5.1). Nor indeed did dbcAMP prevent Ad 5 DNA replication. Thus normal Ad 5 functions appear to continue even in the presence of dbcAMP suggesting once again that Ad 5 can induce a cell cycle by overcoming a cellular cAMP sensitive control point.

When a similar experiment was done using G_1-arrested mouse cells a different result was obtained (Table 5.2). Both Ad 5 DNA replication and Ad 5 induced cellular DNA replication were once again not inhibited by 1.0mM dbcAMP, indeed dbcAMP treated Ad 5 infected cultures showed a higher stimulation of cellular DNA synthesis than the untreated Ad 5 control as was repeatedly obtained in rat cells, but the drug had much less of an effect on serum induced DNA replication than in rat cells. In Table 5.2,

TABLE 5.1

Effect of dbcAMP on induction of cellular and viral DNA synthesis by Ad 5 and cellular DNA synthesis by serum in G_1-arrested rat cells [a]

Inoculum	Addition of dbcAMP	Viral DNA component [b] (c.p.m.)	Cellular DNA component [b] (c.p.m.)	Degree of stimulation [c]
MOCK	-	0	10,300	1.0
10% FCS	-	0	70,040	6.80
10% FCS	+	0	1,007	NS
Ad 5	-	12,803	24,373	2.37
Ad 5	+	6,172	40,276	3.91

a Cells were arrested and infected as described in Chapter 2

b Total labeled DNA from CsCl gradients was analysed into viral and cellular DNA components as described in Chapter 3

c Radioactivity in the cellular DNA component divided by the radioactivity from mock infected cultures in the same fractions. After serum addition or infection 1.0mM dbcAMP was added to cultures and they were then labeled with [^{3}H]thymidine from 36 to 48 h later. DNA was extracted and analysed by CsCl gradient centrifugation

NS no stimulation (c.p.m. less than in mock infected control)

TABLE 5.2

Effect of dbcAMP on induction of cellular and viral DNA replication by Ad 5 and cellular DNA replication by serum in G_1-arrested mouse cells [a]

Inoculum	Addition of dbcAMP	Viral DNA component [b] (c.p.m.)	Cellular DNA component [b] (c.p.m.)	Degree of stimulation [c]
MOCK	-	0	7,336	1.0
10% FCS	-	0	37,524	5.12
10% FCS	+	0	28,495	3.88
Ad 5	-	3,286	23,761	3.24
Ad 5	+	4,085	40,808	5.56

a, b, c As for Table 5.1. After serum addition or Ad 5 infection, 1.0mM dbcAMP was added to the cultures. Cells were labeled with [^{3}H]thymidine from 24 to 44 h later, and total intracellular DNA was extracted and analysed by CsCl gradient centrifugation.

a 30% inhibition of cellular DNA replication was observed, and in other experiments this inhibition ranged from 20% to 40%. Thus mouse fibroblasts are much less sensitive to the effects of dbcAMP than rat cells which emphasises the lack of generality with which cells respond to cAMP regulation of cellular proliferation. Nevertheless, as Ad 5 induced cellular DNA replication was if anything stimulated by dbcAMP in rat and mouse cells, and serum induced DNA replication reduced (by an amount which is dependent on the cell type), these results both support the argument that Ad 5 modifies some controls of cellular proliferation.

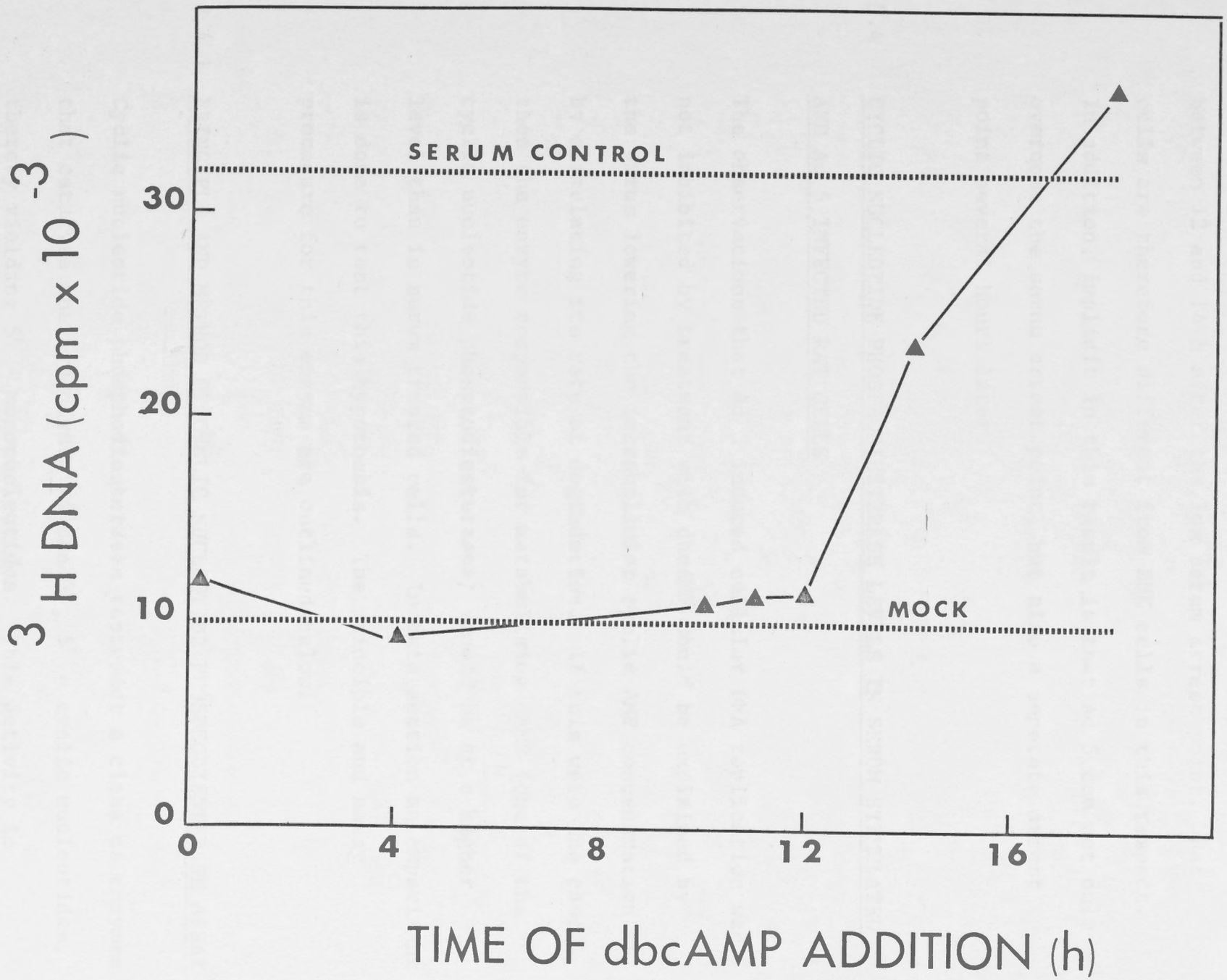
5.3.3 TIME COURSE OF DIBUTYRYL CYCLIC AMP INHIBITION OF RAT CELL PROLIFERATION

The results of double blocking experiments reported by Pardee (1974) suggested that serum starvation and dbcAMP restricted the BHK cell cycle at approximately the same point in G_1. Whether or not serum starvation and dbcAMP also block the rat cell cycle at the same point is not known. In this section an experiment is presented which answers this question.

G_1-arrested rat cells were serum stimulated as previously described and at different times, beginning at the time of serum addition, 1.0mM dbcAMP was added to the cultures. Cells were then labeled with [^{3}H] thymidine from 14 to 26 h later and the incorporated radioactivity was determined. Results of this experiment (Fig. 5.3) showed that dbcAMP prevented entry into DNA

FIGURE 5.3

Time of dbcAMP inhibition of the induction of DNA synthesis in rat cells. Queiscent rat cells were serum stimulated and 1.0mM dbcAMP was added immediately or at the indicated times after serum treatment. Cells were labeled with [^{3}H] thymidine from 14 to 26 h after serum addition and DNA synthesis measured as previously described. The hatched lines represent untreated (no dbcAMP) G_1-arrested (MOCK) and serum stimulated (SERUM CONTROL) cultures.


SERUM CONTROL
MOCK
30
20
10
0
0
4
8
12
16
3 H DNA (cpm x 10 -3)
TIME OF dbcAMP ADDITION (h)

synthesis when added up to 12 h after serum addition. If dbcAMP was added after 12 h (beginning at 14 h, see Fig. 5.3), DNA synthesis was not inhibited. This result therefore suggested that there is a cAMP sensitive proliferation control point between 12 and 14 h after the low serum arrest point. Rat cells are therefore different from BHK cells in this respect. In addition, implicit in this result is that Ad 5 can not only overcome the serum arrest point, but also a separate arrest point several hours later.

5.3.4 CYCLIC NUCLEOTIDE PHOSPHODIESTERASE LEVELS IN SERUM STIMULATED AND Ad 5 INFECTED RAT CELLS

The observations that Ad 5 induced cellular DNA replication was not inhibited by treatment with dbcAMP could be explained by the virus lowering the intracellular cyclic AMP concentration by stimulating its rate of degradation. If this were the case then the enzyme responsible for metabolising cAMP (one of the cyclic nucleotide phosphodiesterases) should be at a higher level than in serum treated cells. In this section an experiment is done to test this hypothesis. The principle and assay procedure for this enzyme are outlined below.

5.3.4.1 PRINCIPLE AND METHOD OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ASSAY

Cyclic nucleotide phosphodiesterases represent a class of enzymes that catalyse the hydrolysis of the 3' , 5' - cyclic nucleotides, thereby yielding 5' - mononucleotides. This activity is measured as the conversion of radioactively labeled cyclic

nucleotide substrates to the labeled nucleotide. In the "batch" assay of Thompson and Appleman (1971) tritiated substrates are converted by phosphodiesterases present in cellular extracts to the respective 5' - mononucleotides (e.g. 5' - AMP). These are then separated from the unconverted substrate by enzymatic conversion to the nucleosides (e.g. adenosine) by the 5' - nucleotidase activity present in snake venom. The nucleosides can then be separated from the charged cyclic nucleotide substrates by precipitation of the latter with an anion-exchange resin, leaving the radioactivity associated with the nucleosides in the supernatant fraction. This can be measured by liquid scintillation spectrometry.

For the experiment described in the next section the following protocol was used. Between 5 and 7 x 10^5 rat cells were seeded into dishes and then made quiescent by incubation in 0.2% BS for 3 days. They were serum stimulated or infected with Ad 5 and then at appropriate times (see Fig. 5.4) cells were washed three times with normal saline (room temperature) and then harvested by scraping. Cells were centrifuged (in a Beckman Model B bench microfuge for 5 minutes), suspended in 1.0ml of 50mM tris buffer, pH 7.4 (0^o) and then sonicated on ice.

The reaction mixture for the phosphodiesterase assay consisted of a mixture of [^{3}H] cyclic AMP (50 µl) and unlabeled cyclic AMP (50 ul of a 4 µM solution) which gave a final substrate concentration of 1 µm (this was used to selectively detect cAMP phosphodiesterase activity; M.M. Appleman, personal

communication). 100 µl of cell extract was added to this reaction mixture and incubated for 30 minutes *, at 37°C in a shaking waterbath. At this time 50 µl of 5' - nucleotidase from snake venom (Sigma) (see Section 2.4 for details of reagents) was added and the reaction mixture was incubated for a further 20 minutes. The reaction was terminated by addition of 1.0ml of an anion-exchange resin slurry (3 parts 3mM acetic acid to 1 part resin), then incubated for 10 minutes, vortexed, and centrifuged (2000g for 5 minutes). A 500 µl aliquot of the supernatant was then removed and the radioactivity determined.

5.3.4.2 CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN SERUM TREATED AND Ad 5 INFECTED RAT CELLS

Quiescent rat cells were serum stimulated or infected with Ad 5 (10 iu/cell) then at appropriate times cells were harvested and the cyclic AMP phosphodiesterase activities were determined using the procedures outlined above (Section 5.3.4.1). This experiment was done in collaboration with Dr. M.M. Appleman (University of Southern California, Los Angeles).

Results from this experiment (Fig. 5.4) showed that 10% FCS caused a small stimulation of cyclic AMP phosphodiesterase activity (up to a maximum of 1.6 fold stimulation; this represented a difference in nearly 1500 cpm of [^{3}H] cyclic

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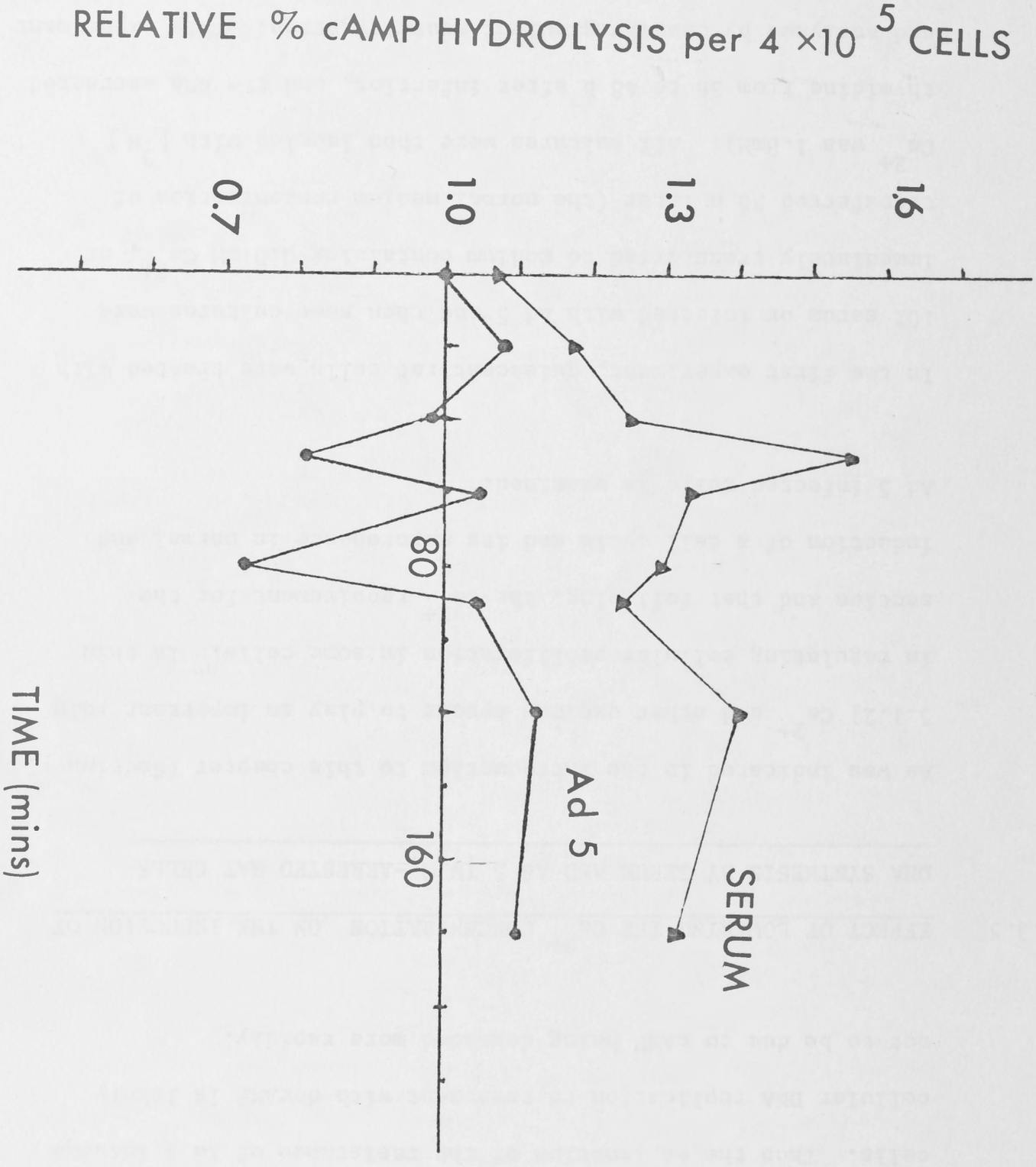
* This time of incubation was chosen after kinetic studies were done showing that substrate conversion was below 25% of the total substrate available to the enzymes (M.M. Appleman, unpublished observations). Longer incubation produced non-linear enzyme kinetics.

FIGURE 5.4

Cyclic AMP phosphodiesterase activity in serum treated and Ad 5 infected rat cells. Quiescent rat cells were serum stimulated or infected with Ad 5 as described in Chapter 2. At indicated times cells were harvested as described in Section 5.3.4.1 and the cyclic AMP phosphodiesterase activity of each culture was determined (see Section 5.3.4.1). For further details of assay procedure refer to Thompson and Appleman (1971) and Evans (1981). The results are expressed as relative % cAMP hydrolysis per 4×10^5 cells taking 1.0 to be the mock infected enzyme activity at each time.


RELATIVE % cAMP HYDROLYSIS per 4 ×10 5 CELLS
0.7
1.0
1.3
1.6
80
160
TIME (mins)
Ad 5
SERUM

AMP hydrolysis) relative to mock infected controls. By contrast Ad 5 caused no significant change in enzyme activity. These data therefore suggest that cAMP is likely to be metabolised at a slower rate in Ad 5 infected cells compared to serum treated cells. Thus the explanation of the resistance of Ad 5 induced cellular DNA replication to treatment with dbcAMP is likely not to be due to cAMP being degraded more rapidly.

5.3.5 EFFECT OF LOWERING THE Ca^{2+} CONCENTRATION ON THE INDUCTION OF DNA SYNTHESIS BY SERUM AND Ad 5 IN G_1-ARRESTED RAT CELLS

As was indicated in the introduction to this chapter (Section 5.1.2) Ca^{2+} and other cations appear to play an important role in regulating cellular proliferation in some cells. In this section and that following, the Ca^{2+} requirement for the induction of a cell cycle and its maintenance in normal and Ad 5 infected cells is examined.

In the first experiment, quiescent rat cells were treated with 10% serum or infected with Ad 5 and then some cultures were immediately transferred to medium containing 0.01mM Ca^{2+}, or transferred 36 h later (the normal medium concentration of Ca^{2+} was 1.8mM). All cultures were then labeled with [^{3}H] thymidine from 36 to 48 h after infection, and the DNA extracted and analysed by density gradient centrifugation. This experiment showed that lowering the Ca^{2+} concentration to 0.01mM prevented entry into DNA synthesis in both serum treated and Ad 5 infected cultures (Table 5.3).

TABLE 5.3

Effect of low Ca^{2+} concentration on the induction of cellular DNA replication by serum and Ad 5 in G_1-arrested rat cells [a]

Inoculum	Time of transfer to low Ca^{2+} medium (h) [d]	Viral DNA component (c.p.m.) [b]	Cellular DNA component c.p.m.) [b]	Degree of stimulation [c]
MOCK	NT	0	1,264	1.0
10% FCS	NT	0	29,577	23.40
10% FCS	0	0	3,238	2.56
10% FCS	36	0	9,042	7.15
Ad 5	NT	3,777	3,062	2.42
Ad 5	0	2,564	866	NS
Ad 5	36	7,248	6,913	5.47

a Rat cells were G_1-arrested by culturing in medium containing 0.2% BS for 3 days prior to infection or serum addition

b Total labeled DNA from CsCl gradients was analysed into viral and cellular DNA components as described in Chapter 3

c Radioactivity in the cellular DNA component divided by the radioactivity from mock infected cultures in the same fractions

d Cells were shifted to low Ca^{2+} medium (0.01mM) at the time of infection or serum addition (0 h) or 36 h later, then labeled from 36 to 48 h

NS no stimulation (c.p.m. less than mock infected control)

NT not transferred

DNA synthesis was also inhibited in serum stimulated cultures which were transferred to low Ca^{2+} medium 36 h after serum addition, but Ad 5 infected cells transferred to low Ca^{2+} medium at this time continued to synthesise cellular DNA at control levels (Table 5.3). These results suggested that a minimum crucial concentration of Ca^{2+} was required for rat cells to enter the DNA synthesis phase of the cell cycle irrespective of the agent initiating the cell cycle (serum or Ad 5). Ca^{2+} also appeared to be required for cellular DNA synthesis to be maintained in normal cells, but Ad 5 appeared to confer resistance to low Ca^{2+} inhibition of continued cellular DNA replication. Thus Ad 5 might overcome another control point of cellular proliferation in a way which is relevant to virus transformed cells being selected in culture (see Freeman et al., 1967).

5.3.6 TIME COURSE OF LOW Ca^{2+} ION EFFECT ON THE INDUCTION OF CELLULAR DNA SYNTHESIS BY Ad 5 IN G_1-ARRESTED RAT CELLS

The results presented in Table 5.3 showed that a low Ca^{2+} concentration inhibited the induction of cellular DNA replication by Ad 5, but once induction had taken place continued cellular DNA replication was unaffected by lowering the Ca^{2+} concentration; that is, at some time point Ad 5 infected rat cells became resistant to the effects of low Ca^{2+}. In this section cells were transferred to low Ca^{2+} medium at different times after infection with Ad 5 in order to approximately determine the time when these cells became resistant to low Ca^{2+} ion effects. This was examined both by gradient centrifugation of [^{3}H]

TABLE 5.4

Time course of low Ca^{2+} ion effect on induction of cellular DNA replication in rat cells [a]

Inoculum	Time of transfer to medium containing 0.01mM Ca^{2+} (h) [d]	Viral DNA component (c.p.m.) [b]	Cellular DNA component (c.p.m.) [b]	Degree of stimulation [c]
MOCK	NT	0	5,108	1.0
Ad 5	NT	18,531	16,774	3.28
Ad 5	0	10,506	4,058	NS
Ad 5	5	11,038	6,852	1.34
Ad 5	10	11,500	4,150	NS
Ad 5	18	11,560	16,090	3.15
Ad 5	24	14,541	13,813	2.70
Ad 5	36	20,922	23,728	4.64

a, b, c As for Table 5.3

d Cells were shifted to medium containing 0.01mM Ca^{2+} at the time of infection (0 h) or at indicated times thereafter All cultures were labeled with [^{3}H]thymidine from 36 to 48 h then intracellular DNA was extracted and centrifuged to equilibrium

NS no stimulation (c.p.m. less than mock infected control)

NT not transferred

thymidine labeled intracellular DNA and by cell cycle analysis using flow cytometry.

Gradient analysis of DNA from Ad 5 infected cells showed that 0.01mM Ca^{2+} inhibited cellular DNA replication up to 10 h after infection but had no effect from 18 h after infection onwards (Table 5.4). Thus some Ad 5 gene product confers resistance to cells from the effects of low Ca^{2+} ions between 10 and 18 h after infection. A similar result was obtained by flow cytometric analysis of Ad 5 infected, low Ca^{2+} treated cells. In this experiment (Table 5.5) cultures treated with 0.01mM Ca^{2+} up to 10 h after infection primarily contained cells with G_1 DNA contents (85%; similar to the mock infected control), whereas untreated Ad 5 cultures, and those treated with low Ca^{2+} ions from 18 h onwards had a reduced proportion of cells in G_1, and a greater proportion with $>G_1$ DNA contents. Thus once again Ad 5 caused cells to become resistant to the inhibitory effects of low Ca^{2+} concentrations between 10 and 18 h after infection. Thus it would appear that Ad 5 overcomes a Ca^{2+} - sensitive control point for cellular proliferation as it does for low serum and cAMP.

TABLE 5.5

Flow cytometric analysis of Ad 5 infected rat cells [a] treated with 0.01mM Ca^{2+}

Inoculum	Time of transfer to 0.01mM Ca^{2+} medium (h) [b]	Approx. % cells with DNA contents corresponding to:	
		G_1	$>G_1$
MOCK	-	88.0	12.0
Ad 5	-	74.0	26.0
Ad 5	0	89.5	10.5
Ad 5	5	89.1	10.9
Ad 5	10	84.9	15.1
Ad 5	18	71.2	28.8
Ad 5	36	69.2	30.8

a Cells were arrested and infected as described in Chapter 2

b After infection cells were shifted immediately to medium containing 0.01mM Ca^{2+} (0 h) or at indicated times thereafter.
48 h after infection all cultures were harvested by trypsinisation and stained with EB and MMC for DNA content analysis by flow cytometry. For details of staining procedure refer to Section 4.2.2. This experiment was done using the FACS IV in Canberra.

5.4 DISCUSSION

The results of experiments reported in this chapter showed that normal rat cell proliferation could be prevented by shifting cells to medium containing a low Ca^{2+} concentration or by treatment with the cAMP analogue dbcAMP. By adding dbcAMP at different times after serum stimulation of G_1-arrested cells, it was found that cells became resistant to treatment with dbcAMP between 12 and 14 h after the low serum arrest point. This observation suggested that there was a cAMP sensitive control point regulating cellular proliferation which was separate from the low serum control point. That sodium butyrate had little or no effect on serum induced DNA replication suggested that the inhibition of DNA synthesis by dbcAMP was not due to hydrolysis of dbcAMP and therefore provided confirmatory evidence for the above interpretation of a cAMP sensitive control point. In contrast to cAMP, Ca^{2+} ions appeared to be continually required for induction and for maintenance of normal cellular DNA replication.

In Ad 5 infected rat cells, viral DNA replication and virus induced cellular DNA replication were both unaffected by dbcAMP at a concentration which completely prevented serum induced DNA replication (see Table 5.1). Thus Ad 5 induces proliferation in rodent cells when normal proliferation is prevented or slowed both by serum starvation (see Chapter 3) and by dbcAMP treatment, suggesting that the virus can overcome more than one proliferation control point. Induction of cellular DNA replication by Ad 5

was however prevented by low Ca^{2+} treatment, but once initiated virus induced cellular DNA replication became insensitive to low Ca^{2+}. This reduced sensitivity to the effects of low Ca^{2+} occurred between 10 and 18 h after infection. Thus at least a pulse of Ca^{2+} is required for initiation of cellular DNA replication irrespective of the initiating agent, but once initiated Ad 5 can maintain cellular DNA replication without continually requiring Ca^{2+}, unlike serum growth factors. Such results suggested that not only are the cellular controls for induction of DNA synthesis altered by Ad 5 infection, but so too are the controls for maintenance of cellular DNA replication.

Examination of the effects of serum and Ad 5 on the cyclic AMP phosphodiesterase from rat cells (the enzyme that degrades cAMP) showed that whilst serum caused small but reproducible increases in the activity of this enzyme Ad 5 had no effect. This experiment (Fig. 5.4) only extended to 3 h after serum treatment or Ad 5 infection, but this time period was determined to be the region over which the greatest increases in enzyme activity were observed (M.M. Appleman, unpublished observations). This result suggested that cAMP probably must be degraded early after serum stimulation in order to prevent cells from becoming arrested 12 to 14 h later. As Ad 5 had no effect on the activity of the cyclic AMP phosphodiesterase it seems unlikely that the resistance of Ad 5 induced cellular DNA replication to dbcAMP can be explained by a higher rate of cAMP metabolism. This lack of enzyme induction by Ad 5 also provides more evidence for altered controls of cellular proliferation. Ad 5 may induce

cellular DNA replication via a different pathway from serum. Other evidence for this interpretation arises from the observation that Ad 5 causes cell cycle progression without inducing the enzyme ornithine decarboxylase (Cheetham and Bellett, 1982), which is induced by serum 3 to 8 h after the low serum arrest point.

The data reported in this chapter (and Chapter 3), and the lack of induction of ornithine decarboxylase by Ad 5, collectively showed that Ad 5 can bypass 4 to 5 events in G_1 essential for normal cell cycle progression, thus shortening the length of the G_1 phase. This interpretation is consistent with the results obtained by Rossini et al, (1979 a, b,)and Pochron et al.,(1980) (see Section 5.1.3). These alterations, in particular the altered Ca^{2+} ion sensitivity, might be relevant to the way in which adenovirus causes cellular transformation.

CHAPTER 6

CELL CYCLE REGULATION BY PROTEIN SYNTHESIS IN NORMAL AND Ad 5 INFECTED RODENT CELLS

6.1 INTRODUCTION

The proliferation rate of animal cells is largely determined by the relative amount of time spent in the G_1 phase of the cell cycle (see Prescott, 1976; see Chapter 1). As was discussed in Chapter 1, in each cell cycle there appears to be an initiation event which regulates entry into the next cell cycle; in its absence cells enter a quiescent state in G_1 (Pardee, 1974). Once this initiation event has taken place cellular proliferation appears to be comparatively unaffected by growth conditions (Smith and Martin, 1973), thus major growth regulation occurs by the cyclic occurrence of the initiation event before the onset of DNA synthesis. The experiments of Schneiderman et al., (1971) and Rossow et al., (1979) (and others) provided evidence that this cell cycle initiation occurred through synthesis of a class of specific initiator proteins which are labile and serum dependent (Rossow et al., 1979). These studies and others were discussed in detail in Chapter 1. In the studies of Rossow et al., (1979) for example, it was shown that concentrations of cycloheximide that inhibit protein synthesis in balb/c 3T3 mouse cells by up to 70% specifically delay transit through G_1 prior to the "restriction point" (Pardee, 1974). These results were explained by supposing that labile (short half-life) initiator proteins must be synthesised to threshold levels before progression to DNA synthesis can continue, and that external growth conditions affected synthesis of these proteins. Other evidence that is consistent

with this hypothesis comes from several observations that DNA synthesis declines very rapidly after inhibition of protein synthesis by cycloheximide (for example, Weintraub and Holtzer, 1972; Searle and Simpson, 1975). These data could also be partly explained by cycloheximide directly inhibiting synthesis of proteins required continually in the DNA replication complex. In Hela cells cycloheximide allows normal replicative synthesis to occur, albeit at a slowed rate compared to controls, and 4s and 5s Okazaki fragments are synthesised and processed to high molecular weight DNA. Furthermore, replication forks identified in CsCl are selectively labeled with [^{3}H] thymidine in both untreated and cycloheximide treated cells (Searle and Simpson, 1975). However, there does appear to be some defect in short nascent strand maturation in these cells as 21s fragments (intermediate in size between Okazaki fragments and mature DNA) accumulate in cycloheximide treated cells. Thus cycloheximide does have some effect on the maturation of newly synthesised DNA, and therefore it may have more than one effect on cellular DNA synthesis.

Delayed entry into DNA synthesis by inhibition of protein synthesis with cycloheximide could be explained by (i) a delay in synthesis of a particular initiator protein to an effective level, such as is suggested by Schneiderman et al., (1971) and Rossow et al., (1979), or (ii) by a general delay in progression through the G_1 phase to the onset of DNA synthesis, by inhibiting synthesis of many proteins. At a high enough concentration of cycloheximide, such as that used

in Chapter 3 (1.0 µg/ml; see Table 3.7), a general inhibition of cell cycle progression does occur and cells therefore accumulate in G_1 (see Table 3.7) as well as in other stages of the cell cycle. However, partial inhibition of protein synthesis (up 0.1 µg/ml) slows down cell cycle progression but does not completely prevent it. Using "normal" 3T3 mouse fibroblasts which had been made quiescent by serum starvation, Brooks (1977) studied the kinetics of entry into DNA synthesis in untreated and cycloheximide (up to 0.1 µg/ml) treated cells after the addition of fresh serum growth factors. Following readdition of serum and after a lag of 14 hours cells entered S phase (DNA synthesis) with first-order kinetics. This kind of cell cycle progression after stimulation was consistent with a single, rate-limiting random event (or transition, Smith and Martin, 1973) regulating progression. The addition of low concentrations of cycloheximide (0.033 - 0.1 µg/ml) at any time after the lag phase brings about a rapid reduction (within 1 - 2 hours) of the rate constant for entry into S phase by an amount that is proportional to the inhibition of [^{3}H]leucine incorporation. These results suggest that the transition probability depends on the continuous synthesis of a short half-life (labile) protein regulating entry into DNA synthesis (i.e. a single regulatory event), and therefore not a general decline in progression to DNA synthesis. The data also suggested that this rate-limiting event occurs within 2 hours of DNA synthesis, which is later than that suggested by Rossow et al., (1979).

Tumor and transformed cells are defective in some aspects of growth control regulation (Ponten, 1976). Many such cells when in tissue culture are not so readily arrested by high density (Baserga, 1976), low serum concentration (Holley, 1975) or drugs (Pardee and James, 1975; Dubrow et al., 1979). With this background, Medrano and Pardee (1980) investigated the effects on G_1 transit time of partially inhibiting protein synthesis with cycloheximide in variously transformed tumorogenic cells. Using several transformed cell lines of 3T3 mouse cells these workers showed that there was a gradation of sensitivity to cycloheximide treatment which was related to the degree of tumorogenicity. For example, their SV-A31 cells, which are SV 40 transformed mouse cells, showed no change in flow cytometry determined DNA histograms after treatment with cycloheximide up to 0.1 µg/ml.* This concentration caused a selective increase in the G_1 population and a decline in S and G_2 cells in the 3T3 cell controls and in the nontumorogenic 3T6 cell clones derived from the 3T3 cells. Other cells were tested such as the chemically transformed T30-4 hamster cell line which once again appeared to be insensitive for growth after treatment with 0.1 µg/ml cycloheximide.

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* This concentration of cycloheximide causes 70-80% inhibition of protein synthesis in untransformed 3T3 mouse cells (Rossow et al., 1979).

A similar lack of sensitivity to cycloheximide has also been reported for SV40-transformed human cells (Ide et al., 1979). These workers showed that short pulses of high concentrations of cycloheximide (up to 3-5 hours) caused severe inhibition of Hela cell DNA synthesis (>65% with 0.5 µg/ml by 1.5 hours after cycloheximide addition) but had no effect on the DNA synthesis of SV40-transformed Hela cells (SV80). Under the same conditions total protein synthesis was inhibited in both cell types, and this was greater in SV80 cells (31% of controls for Hela cells; 18% of controls for SV80 cells by 1.5 h after 0.5 µg/ml cycloheximide addition). The DNA from cycloheximide treated SV80 cells appeared to be normal as the [^{3}H] radioactivity was localised in the nucleus; was resistant to alkaline hydrolysis; and was completely solubilised by acid hydrolysis. Ide et al., (1979) reported that this observed resistance to cycloheximide in the DNA synthesis of SV40-transformed human cells was also the case for several SV40 transformed hamster cell lines. In addition, in hamster cells transformed with a ts gene A mutant of SV40 (their CHLA 239L1) they found that cellular DNA synthesis was resistant to cycloheximide at the permissive temperature for expression of the gene A product (T antigen), but this resistance was lost when cells were cultured at the nonpermissive temperature. Thus cycloheximide resistant cellular DNA replication appears to be controlled by the SV40 T antigen; the protein responsible for causing cellular mitogenesis in SV40 infected cells (Graessmann et al., 1980; see Chapters 3 and 4 for other references).

It is interesting in this context that protein synthesis might also in some cases be required to maintain the transformed phenotype of both virally (kirsten murine sarcoma virus) and chemically (N-methyl-N'-nitro-N-nitrosoguanidine) transformed cells (Cho and Rhim, 1979). These workers showed that treatment of cells isolated from a human osteosarcoma with 0.08 µg/ml cycloheximide, a concentration having no effect on growth of these cells, caused the phenotype of these cells to become more "normal". That is, they exhibited density-dependent growth control and they appeared flattened and fibroblast-like. In addition, antigens expressed on the surface of these transformed cells became undetectable after cycloheximide treatment. Thus from these studies it would seem that continual protein synthesis is required for maintenance of the transformed state and implies the existence of one or more "transforming genes" which are also growth regulatory.

The experiments discussed above and in previous chapters, as well as results presented in previous chapters, all emphasised the differences in growth properties of transformed and tumor cells compared to normal cells. Results with SV40 (see Section 4.1.2 and above) and with adenovirus (see Chapters 3 and 4) strongly suggested that the viral transforming genes are also responsible for altering normal cell growth controls (gene A for SV40; early region 1A for adenovirus). In addition, the SV40 gene A product (T protein) induces cellular DNA replication through some mechanism which is not affected

by normally inhibitory concentrations of cycloheximide (Ide et al., 1979). Furthermore, the DNA synthesis of other virally and chemically transformed cells also appears to be resistant to cycloheximide treatment. Such results suggest that virally coded growth regulatory gene products are either very stable or are synthesised in a way which is not affected by cycloheximide treatment. It seemed possible that this resistance to cycloheximide might be a particular feature of those tumor virus gene products which are responsible for inducing cellular DNA synthesis, and thus of interest to determine whether a similar situation existed with respect to adenovirus induced cellular DNA synthesis. Results reported in Chapter 3 (Section 3.3.6) showed that a high concentration of cycloheximide (1.0 µg/ml) prevented Ad 5 induced cellular DNA synthesis but allowed continued synthesis of viral DNA, provided the drug was added after the initiation events had taken place. In this chapter low concentrations of cycloheximide, sufficient to partially inhibit protein synthesis, are used to further dissect the control mechanisms of adenovirus induced and normal cellular DNA replication. More evidence is provided of abnormalities in the control mechanisms of cellular DNA replication when adenovirus is used as the initiating agent, and some data suggesting that adenovirus early gene products, like SV40 T protein, have a greater resistance to cycloheximide than cellular protein synthesis.

6.2 METHODS

For the experiments reported in this chapter DNA synthesis was measured by the incorporation of radioactivity from [^{3}H] thymidine into trichloracetic acid precipitable material as described in Section 2.2.1. Flow cytofluorimetric measurements were carried out as described previously (Section 4.2.2) as well as measurement of viral gene expression by the indirect fluorescent antibody method (Section 2.2.4).

6.3 RESULTS

6.3.1 EFFECT OF CYCLOHEXIMIDE ON THE INDUCTION OF CELLULAR DNA REPLICATION BY SERUM AND Ad 5 IN G_1-ARRESTED CELLS

Quiescent mouse embryo cells were serum stimulated or infected with wild-type Ad 5 as described in previous chapters. Cycloheximide was then added to the culture medium at different concentrations in the range 0.006 to 3.0 µg/ml and 24 h later cells were labeled with [^{3}H] thymidine for 24 h. Cells were then harvested and the incorporated radioactivity was determined. Results of this experiment (Fig. 6.1) showed the surprising response that far from being inhibited by cycloheximide, incorporation of [^{3}H] thymidine into the DNA of serum stimulated cells was increased by cycloheximide. Fig. 6.1 showed a cycloheximide dependent stimulation of cellular DNA synthesis up to 0.05 µg/ml cycloheximide which gave about two-fold higher incorporation than that in serum stimulated cells without

FIGURE 6.1

DNA synthesis in G_1-arrested mouse cells infected with Ad 5 or treated with 10% FCS in the presence of cycloheximide. Mouse cells were serum arrested (see Chapter 2) then infected with Ad 5 or treated with 10% serum. At the time of infection or serum addition cycloheximide was added to the culture medium at indicated concentrations and cultures were labeled with [^{3}H] thymidine from 24 h to 48 h to measure DNA synthesis. Cells were then harvested and incorporated radioactivity was counted using liquid scintillation spectrometry.

The hatched line (labeled MOCK) represents the total incorporated radioactivity in MOCK infected G_1-arrested cultures.

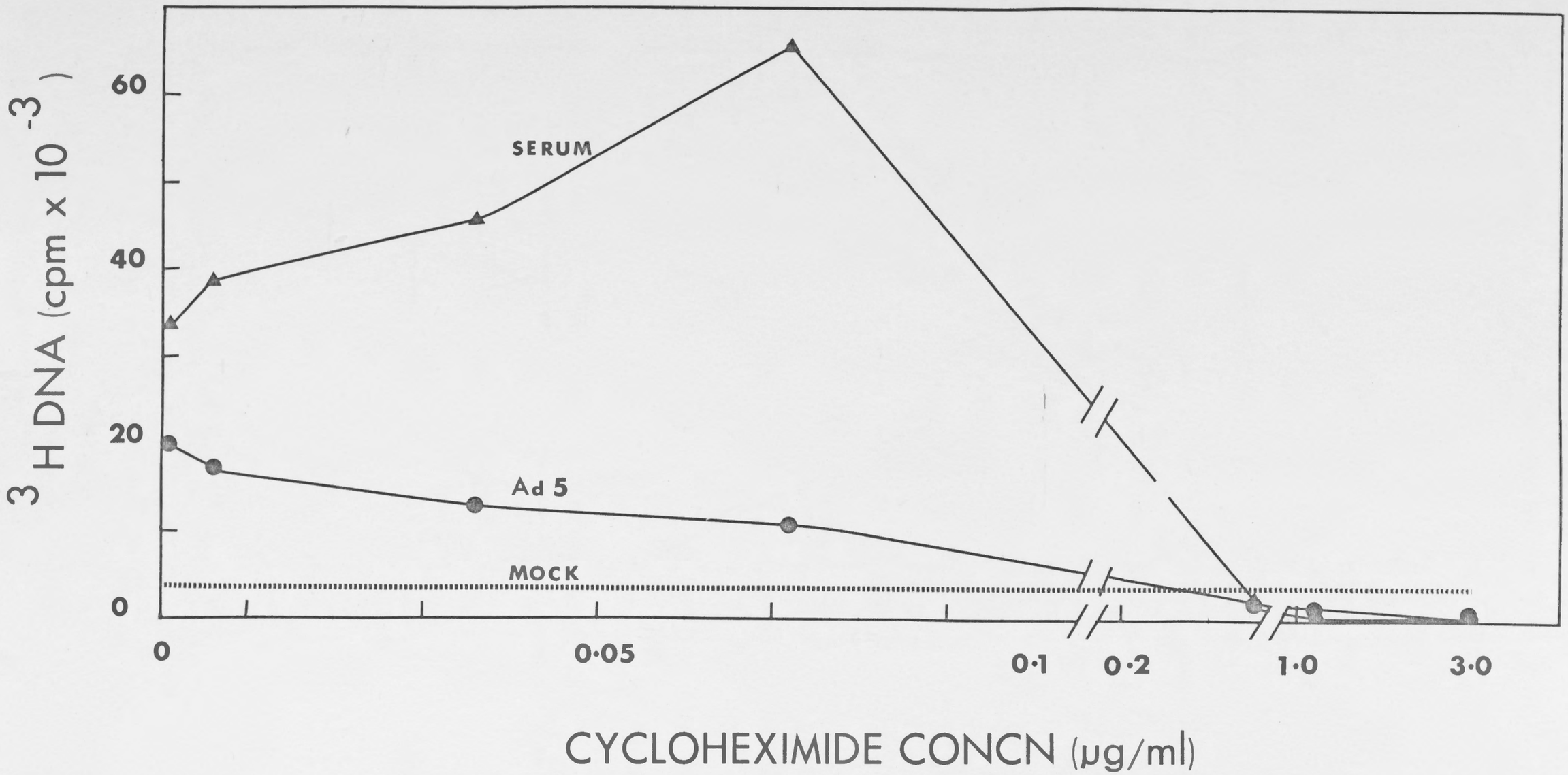
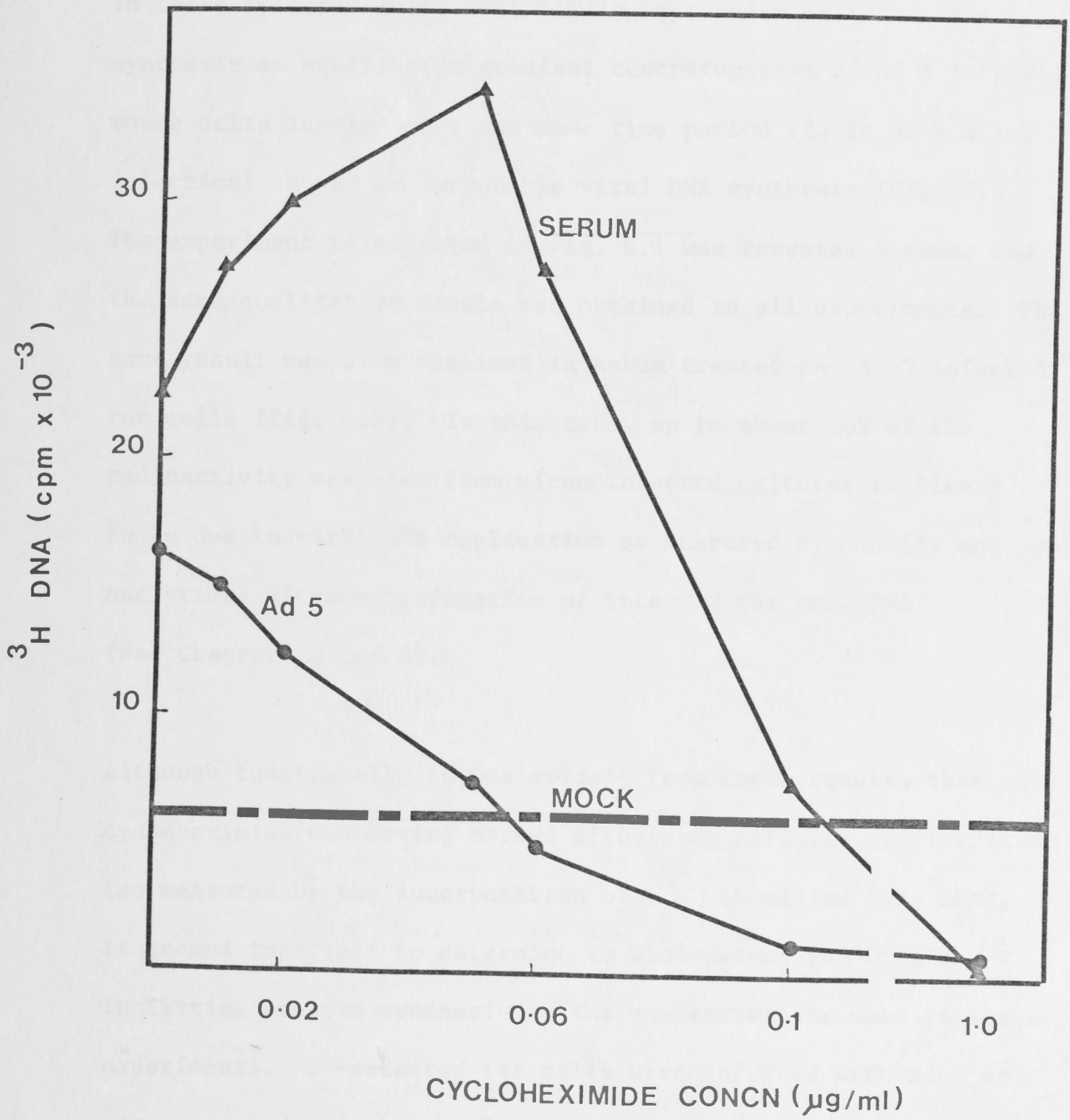

^{3}H DNA (cpm x 10^{-3})
60
40
20
0
SERUM
Ad 5
MOCK
0
0·05
0·1
0·2
1·0
3·0
CYCLOHEXIMIDE CONCN (µg/ml)

FIGURE 6.2

Effect of cycloheximide on DNA synthesis induced in G_1-arrested rat cells by serum treatment (10% FCS) or infection with Ad 5. Rat cells were infected with Ad 5 or serum treated and then labeled with [^{3}H] thymidine from 24 to 48 h later. DNA synthesis was measured by the incorporation of radioactivity after washing in 10% trichloracetic acid. The hatched line represents DNA synthesis from mock infected (G_1-arrested) cells.


30
20
10
SERUM
Ad 5
MOCK
0·02
0·06
0·1
1·0
^{3}H DNA (cpm x 10^{-3})
CYCLOHEXIMIDE CONCN (µg/ml)

cycloheximide. At greater concentrations of cycloheximide there was an inhibition of incorporation which by 1.0 µg/ml was almost totally abolished. In contrast, the DNA replication induced by Ad 5 was inhibited by cycloheximide in a concentration-dependent manner (Fig. 6.1). The total incorporated radioactivity measured in these infected cells most likely represents cellular DNA synthesis as equilibrium gradient centrifugation of Ad 5 infected mouse cells labeled over the same time period (24 to 48 h after infection) showed no detectable viral DNA synthesis (Fig. 3.2). The experiment illustrated in Fig. 6.1 was repeated 3 times and the same qualitative result was obtained in all experiments. The same result was also obtained in serum treated and Ad 5 infected rat cells (Fig. 6.2). In this case, up to about 20% of the radioactivity measured from virus infected cultures is likely to be due to viral DNA replication as measured by density and analytical ultracentrifugation of infected rat cell DNA (see Chapters 3 and 4).

Although functionally it was evident from these results that cycloheximide was having marked effects on cellular proliferation (as measured by the incorporation of [^{3}H] thymidine into DNA), it seemed important to determine to what extent the drug was inhibiting protein synthesis at the concentrations used in these experiments. G_1-arrested rat cells were infected with Ad 5 or serum treated and then cycloheximide was added to cultures at indicated concentrations and labeled with [^{3}H] lysine from 30 to 48 h later. Results from this experiment (Table 6.1) showed that cycloheximide did indeed inhibit protein synthesis (as measured by inhibition of [^{3}H] lysine incorporation) in these

TABLE 6.1

Effect of Cycloheximide on Total Protein Synthesis in serum treated and Ad 5 infected rat cells [a]

Inoculum	Cycloheximide Concentration (µg/ml)	% incorporation of [^{3}H] lysine (labeling was 30 to 48 h) [b]
Serum	0	100
"	0.01	72
"	0.05	59
"	0.10	28
"	0.50	11
Ad 5	0	100
"	0.01	ND
"	0.05	46
"	0.10	33
"	0.50	9

a Cells were G_1-arrested by culturing in 0.2% BS for 3 days prior to infection or serum stimulation

b Acid-insoluble radioactivity incorporated into serum stimulated or Ad 5 infected cultures treated with cycloheximide was calculated as % [^{3}H]lysine incorporated into drug treated cultures, taking radioactivity in untreated control as 100%

ND not done

cells and this was much the same for both serum treated and Ad 5 infected cultures. At 0.05 µg/ml protein synthesis was reduced 40 to 50% and at 0.5 µg/ml it was reduced to <10% of untreated control values (Table 6.1). This result in conjunction with those presented above (Figs. 6.1 and 6.2) showed that when total protein synthesis is inhibited about 50% with cycloheximide (0.05 µg/ml), incorporation of [^{3}H]thymidine into DNA is stimulated in serum treated cultures but inhibited in Ad 5 infected cultures. However, when protein synthesis is markedly reduced (to <10% of untreated controls) DNA synthesis is inhibited in both serum treated and virus infected cultures.

The most striking observation from the results presented in this section is the different (and opposite) effects low concentrations of cycloheximide have on serum induced and Ad 5 induced cellular DNA replication. Up to 0.05 µg/ml cycloheximide causes a stimulation of [^{3}H]thymidine incorporation into serum treated cells but inhibits incorporation in Ad 5 infected cells. There would seem to be several possible explanations for these results.

Firstly, the inhibition of [^{3}H]thymidine incorporation by cycloheximide in Ad 5 infected cells might be due to cycloheximide inhibiting adenovirus protein synthesis to a greater extent than it does cellular proteins. This might be due to an inhibition of synthesis of one or more of the polypeptides coded for by early region 1A (see Chapter 4).

A second possibility is that Ad 5 might be inducing cellular DNA synthesis using a mechanism which is different from the way serum growth factors induce DNA synthesis. Results presented in Chapter 3 (see Table 3.7) suggested that Ad 5 induced cellular DNA replication probably required continuous synthesis of a cellular protein for its maintenance. Such a protein might be utilised by Ad 5 for maintaining DNA synthesis but not by serum factors, and would be more sensitive to cycloheximide treatment than proteins required to initiate and maintain DNA synthesis in uninfected cells. Thus as the ~~co~~ncentration of cycloheximide is increased, a dose dependent inhibition of DNA synthesis would occur. Such an interpretation is supported by the results presented in the previous chapter which showed that adenovirus was able to induce and maintain a cell cycle by overcoming normal growth control regulatory mechanisms.

For serum stimulated cells, the unexpected observation that low concentrations of cycloheximide stimulated DNA synthesis could be due to an artifact of the assay system used for measuring DNA synthesis. That is, cycloheximide might stimulate a higher specific incorporation of [^{3}H] thymidine into DNA whilst the rate of DNA synthesis is in fact no different from the untreated (no cycloheximide) control. Another possibility is that cycloheximide inhibits synthesis of a "governor" protein (repressor) which regulates the rate of DNA synthesis. In the absence of this protein the rate of DNA synthesis (or the rate of entry into DNA synthesis) is increased and "super-induction" occurs. A third possibility is that low concentrations of

cycloheximide could be delaying entry into DNA synthesis so that by 48 h after serum addition and drug treatment, the drug treated cells would be in mid S phase, whilst the control cells would be completing DNA synthesis and entering the G_2 phase of the cell cycle. Such a situation would give an apparent stimulation of DNA synthesis under the conditions used in my experiments.

These possibilities are investigated in detail in the following sections of this chapter.

6.3.2. CELL CYCLE PROGRESSION IN SERUM STIMULATED MOUSE CELLS AFTER CYCLOHEXIMIDE TREATMENT

If serum stimulated, cycloheximide treated cells really contained a greater proportion in the DNA synthesis phase (S phase) of the cell cycle at 48 h, then analysis of drug treated cells by flow cytometry should show a greater proportion in S phase relative to serum stimulated controls. Such an experiment would exclude the possibility that the apparent stimulation of DNA synthesis by cycloheximide is an artifact of the [^{3}H] labeling procedure for measuring DNA synthesis. Such an experiment is reported in this section.

G_1-arrested mouse cells were serum stimulated and then treated with cycloheximide at different concentrations and 48 h later they were harvested and stained for flow cytometry as described in Section 4.2.2. Results of this experiment (Table 6.2) showed once again that cycloheximide caused an apparent stimulation of cells in the DNA synthesis phase of the cell cycle (S phase) at concentrations from 0.006 µg/ml to 0.1 µg/ml. Although there

TABLE 6.2

Cell Cycle progression in Serum stimulated mouse [a] cells after Cycloheximide Treatment

Inoculum	Number of cells analysed(b)	Cycloheximide Concentration (µg/ml)	Approximate % cell cycle stages at 48 h after addition of fresh serum (±) cycloheximide		
			G_1	S	G_2 + M
MOCK	9,833	0	81.7	9.5	8.7
Serum	14,976	0	68.6	16.4	14.8
Serum	13,143	0.006	64.2	22.5	13.1
"	13,130	0.01	63.7	22.0	14.2
"	11,557	0.02	67.0	20.5	12.3
"	11,054	0.05	68.0	20.2	12.6
"	18,002	0.10	54.0	31.6	14.3
"	13,964	1.0	81.2	8.2	10.5

a Cells were arrested and serum stimulated as described in Chapter 2

b Cells were harvested by trypsinisation at 48 h after serum stimulated, and then stained with EB and MMC for analysis of DNA content by flow cytometry. For details of staining procedure refer to Section 4.2.2.

are more cells in S phase in cycloheximide treated cultures compared to the control, the only really marked difference between drug treated and control cultures occurred at 0.1 µg/ml cycloheximide which was inhibitory in previous experiments (Figs. 6.1 and 6.2). Experiments to be described later suggest that this is probably because by 48 h after serum addition control cells and those treated with very low concentrations of cycloheximide are completing or have completed S phase whereas those treated with 0.1 µg/ml are still in early S phase (see Fig. 6.3). The result however does not alter the general conclusion that, as was obtained with [^{3}H] thymidine labeling of DNA, cycloheximide apparently stimulates cellular DNA synthesis at low concentrations. By 1.0 µg/ml cycloheximide cell cycle progression was prevented and the cell cycle kinetic parameters of these cells were virtually identical to the G_1-arrested control cells (Mock). At the same time as there was an increase in S phase cells, there was a concomitant decline in G_1 cells with no apparent effect on the G_2 population at this time. This experiment therefore confirms that there is a real stimulation of DNA synthesis by cycloheximide in serum stimulated cells and it is not an artifact of the labeling procedure used for the results presented in Figs 6.1 and 6.2. These results also suggest that the major effect of cycloheximide is probably on recruitment of cells from G_1 into S phase, rather than an effect on the rate of cellular DNA replication.

6.3.3 ADENOVIRUS EARLY ANTIGEN EXPRESSION AFTER TREATMENT WITH CYCLOHEXIMIDE

To test the possibility that cycloheximide was causing a decline in Ad 5 induced cellular DNA replication by inhibiting early viral protein synthesis, the effect of cycloheximide on early viral protein expression was measured using the indirect fluorescent antibody technique. G_1-arrested mouse cells grown on glass coverslips were infected with Ad 5 wild-type and then cycloheximide was added at different concentrations to the culture medium. 48 h after infection, coverslips were fixed and stained with early viral P and ALP antibodies then conjugated with fluorescein isothiocyanate. P antiserum reacts with the T antigens (from early regions 1A and 1B) (Russell et al., 1967) and with the DNA binding protein, and ALP antiserum reacts with early viral proteins other than the DNA binding protein (see Section 2.2.5).

Results of this experiment are expressed as the percentage of cells that showed positive fluorescence (see Table 6.3). The coverslips were counted by two people other than myself and the same results were obtained. From these results, it appeared as though viral early protein synthesis was not inhibited by cycloheximide up to a concentration of 0.05 µg/ml detected using two different antibodies, although total protein synthesis was reduced to approximately 1/2 of control values (Table 6.1). Indeed there appeared to be more positively fluorescent cells in the cycloheximide treated cultures than in the untreated control (see Table 6.3).

TABLE 6.3

Ad 5 Early Protein Expression after Treatment of infected mouse cells[a] with Cycloheximide

Inoculum	Cycloheximide Concentration (µg/ml)	% cells staining positive with P antiserum [b]	% cells staining positive with ALP antiserum [c]
MOCK	0	0	0
Ad 5 control	0	57.2	37.3
Ad 5	0.01	63.5	46.2
Ad 5	0.02	72.2	45.0
Ad 5	0.05	65.1	34.0

a G_1-arrested mouse cells were infected with wild-type Ad 5 with 10 iu/cell as described in Section 2.1.4

b The indirect fluorescent antibody technique used for staining these cells is described in detail in Section 2.2.4

c Preparation of ALP antiserum is described in Section 2.2.5

The results from this experiment suggested that the effects of cycloheximide in Ad 5 infected cells are likely to be on the synthesis of some cellular protein(s) which are required for Ad 5 to induce or maintain cellular DNA synthesis but might not be required to induce or maintain DNA synthesis by serum growth factors, as the results with cycloheximide in serum treated cells are different from Ad 5 infected cells (see previous 2 sections). It is still possible however that some early viral protein is inhibited by cycloheximide but this is not detected by either of the antibodies (P or ALP) used in this experiment as neither antiserum is specific for early region 1A polypeptides, which are probably required for inducing cell cycle alterations (see Chapter 4). A second possible caveat in the above interpretation is that the technique used in this experiment might not be sensitive enough to detect a reduction in viral specific protein synthesis until it is almost completely inhibited, as this method does not detect differences in intensity of fluorescent labeling. This could probably be examined better using spectrofluorimetric analysis of surface viral antigens.

Taken together, but with the reservations just mentioned, the results presented so far in this chapter suggest that low concentrations of cycloheximide either increase the rate of cellular DNA replication in serum stimulated cells, or delay entry into DNA synthesis from G_1 such that 48 h later (when the above experiments were assayed) there are more cells synthesising DNA than in the untreated controls. Ad 5 however might induce cellular DNA replication through a different

mechanism, as was suggested from results presented in the previous chapter, and those with Ad 2 reported by Pochron et al.,(1980). The above explanation concerning serum stimulated cells is examined in the next section.

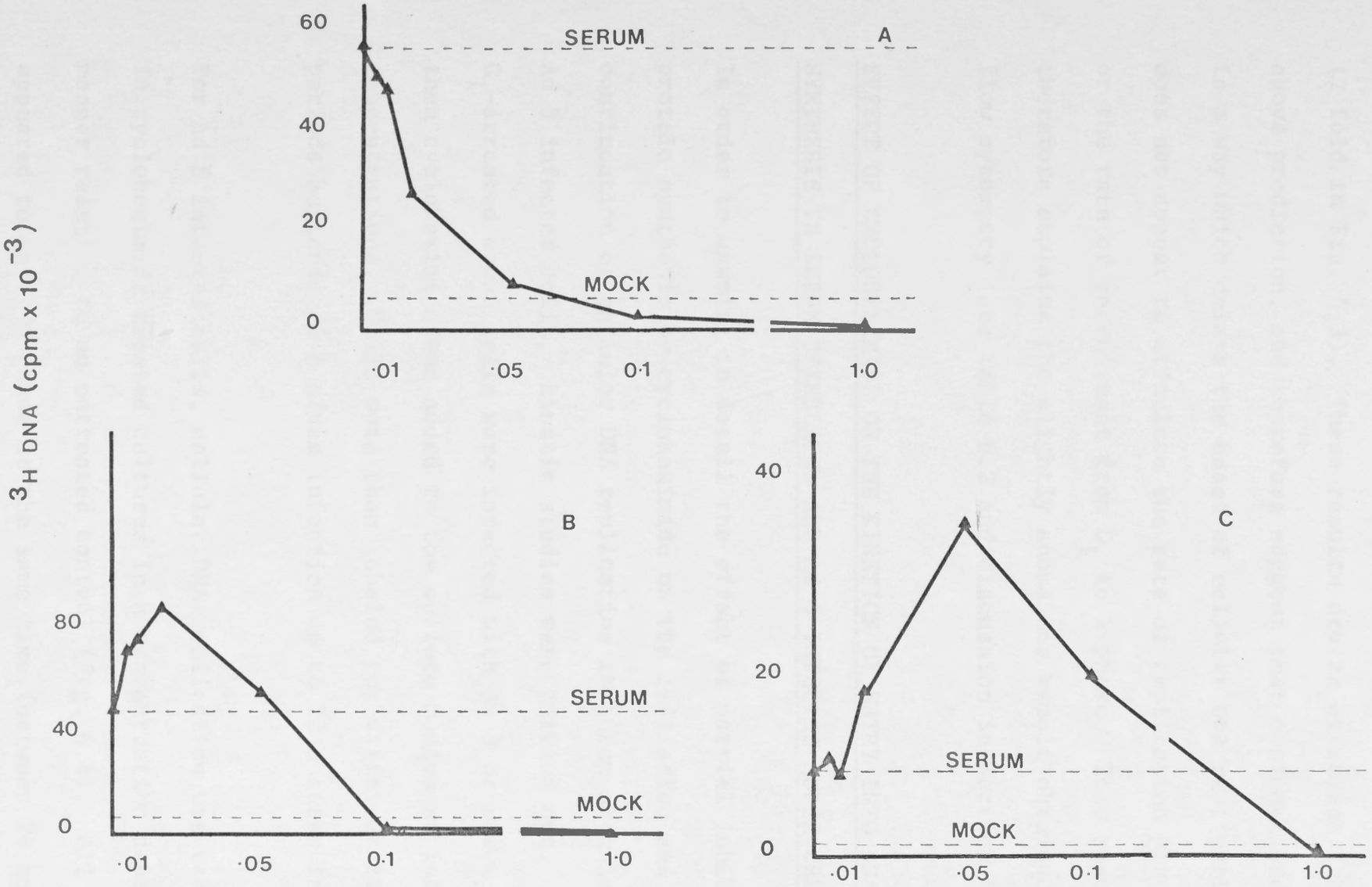
6.3.4 CYCLOHEXIMIDE DELAYS THE G_1 TO S PHASE TRANSITION IN SERUM STIMULATED MOUSE CELLS

If cycloheximide is delaying entry into DNA synthesis (S phase) from G_1 then pulse-labeling cells with [^{3}H] thymidine early after stimulation with serum (from 12 h after serum stimulation) should show a dose dependent decline in DNA synthesis relative to untreated controls, as fewer cells would be entering S phase. By 48 h, however, most untreated cells would have completed their first round of DNA synthesis, but cycloheximide treated cultures would still be predominantly in S phase, showing therefore an apparent stimulation of DNA synthesis.

Results from this experiment showed that cycloheximide reduced incorporation of [^{3}H] thymidine in serum stimulated mouse cells labeled from 12 to 24 h after serum addition, but stimulated incorporation in cells labeled from 24 to 36 h or 36 to 48 h (Fig. 6.3). Untreated serum stimulated cells (no cycloheximide) are probably in mid- to late S phase over 12 to 24 h whereas cycloheximide treated cells are still blocked in the G_1 phase. Thus incorporation is lower in drug treated cultures. By 24 to 36 h however the controls are probably completing DNA synthesis whereas cultures treated with up to 0.05 µg/ml are probably mostly in S phase, thus an apparent stimulation of [^{3}H] thymidine incorporation is observed. Cultures treated with

FIGURE 6.3

Cycloheximide delays entry into S phase in serum stimulated mouse cells. Cells were made quiescent by culturing for 3 days in 0.2% BS and then stimulated into DNA synthesis (S phase) by addition of 10% FCS. Cells were then labeled with [^{3}H] thymidine from 12 to 24 h (A), 24 to 36 h (B) and 36 to 48 h (C) after addition of 10% FCS (SERUM). The hatched lines represent the level of DNA synthesis in a mock infected culture (MOCK) and a serum stimulated culture (SERUM) which were not treated with cycloheximide.


60
40
20
0
SERUM
A
MOCK
·01
·05
0·1
1·0
^3H DNA (cpm x 10^{-3})
80
40
0
SERUM
B
MOCK
·01
·05
0·1
1·0
40
20
0
SERUM
C
MOCK
·01
·05
0·1
1·0
CYCLOHEXIMIDE CONCN (ug/ml)

0.1 μg/ml are now predominantly in S phase (probably early to mid- S phase) thus they too show increased [^{3}H] thymidine incorporation relative to the untreated control (2 fold in Fig. 6.3). These results are in agreement with the above prediction, and therefore suggest that cycloheximide acts in a way which delays the onset of cellular DNA replication and does not appear to stimulate the rate of replication per se, or the rate of recruitment from G_1 to S phase. This result therefore explains the slightly anomalous result obtained with flow cytometry (see Table 6.2 and discussion in Section 6.3.2).

6.3.5 EFFECT OF CYCLOHEXIMIDE ON THE KINETICS OF ENTRY INTO DNA SYNTHESIS IN SERUM STIMULATED AND Ad 5 INFECTED MOUSE CELLS

In order to examine in detail the effect of partial inhibition of protein synthesis by cycloheximide on the initiation and continuation of cellular DNA replication in serum stimulated and Ad 5 infected cells, kinetic studies were carried out. G_1-arrested mouse cells were infected with Ad 5 or serum treated, then cycloheximide was added to the culture medium at indicated concentrations. Cells were then labeled for different time periods beginning 6 h after infection up to 72 h after infection.

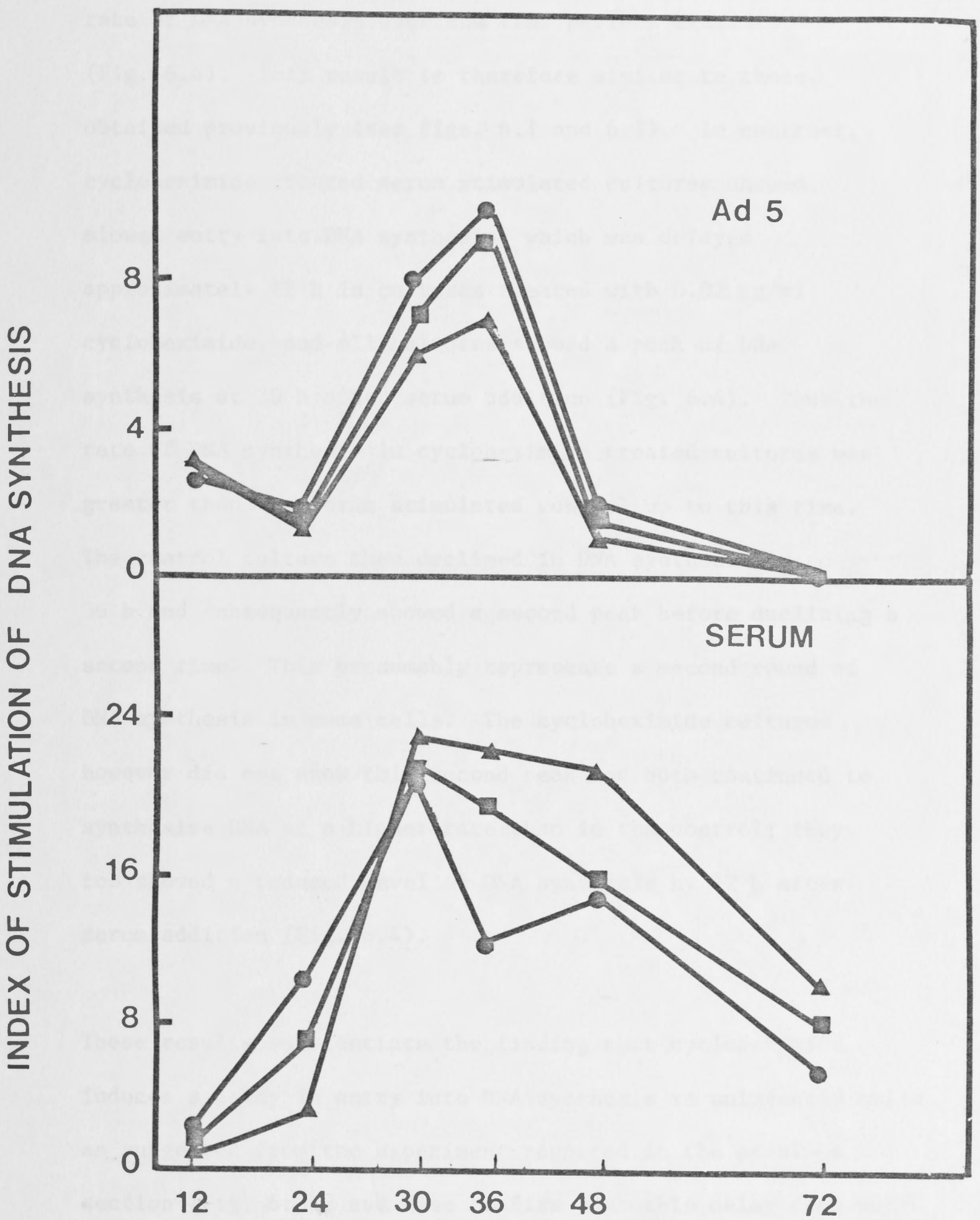
For Ad 5 infected cells, cellular DNA replication was reduced in cycloheximide treated cultures in a concentration dependent manner relative to an untreated control (Fig. 6.4). All cultures appeared to enter S phase at the same time (between 24 and 30 h after infection) and all showed a peak of DNA synthesis at 36 h

FIGURE 6.4

Effect of cycloheximide on the kinetics of entry into S phase from G_1 in serum stimulated and Ad 5 infected mouse cells. Quiescent mouse cells were infected with Ad 5 or stimulated with serum (10% FCS) as previously described. Cycloheximide was then added to cultures and cells were labeled with [^{3}H] thymidine. The times indicated on this figure refer to the end of a labeling period. Thus 12 h represents cells labeled from 6 to 12 h; 24 h represents cells labeled from 12 to 24 h after serum addition; and so on. The index of stimulation (ordinate) is a measure of stimulation calculated by dividing the radioactivity (cpm) in Ad 5 infected or serum stimulated cultures by the radioactivity in a mock infected culture.

Symbols:

- ● Untreated (no cycloheximide) controls
- ■ 0.01 µg/ml cycloheximide
- ▲ 0.02 µg/ml cycloheximide


Ad 5
SERUM
INDEX OF STIMULATION OF DNA SYNTHESIS
0
4
8
0
8
16
24
12
24
30
36
48
72
LABELING TIMES (h)

after infection, but drug treated cells showed a slowed rate of DNA synthesis over the time periods examined (Fig. 6.4). This result is therefore similar to those obtained previously (see Figs. 6.1 and 6.2). In contrast, cycloheximide treated serum stimulated cultures showed slowed entry into DNA synthesis, which was delayed approximately 12 h in cultures treated with 0.02 µg/ml cycloheximide, and all cultures showed a peak of DNA synthesis at 30 h after serum addition (Fig. 6.4). Thus the rate of DNA synthesis in cycloheximide treated cultures was greater than the serum stimulated control up to this time. The control culture then declined in DNA synthesis up to 36 h and subsequently showed a second peak before declining a second time. This presumably represents a second round of DNA synthesis in some cells. The cycloheximide cultures however did not show this second peak and both continued to synthesise DNA at a higher rate than in the control; they too showed a reduced level of DNA synthesis by 72 h after serum addition (Fig. 6.4).

These results substantiate the finding that cycloheximide induces a delay in entry into DNA synthesis in uninfected cells as suggested from the experiment reported in the previous section (Fig. 6.3), and also confirm that this delay does not occur when Ad 5 is used to initiate DNA replication. As well as delaying entry into DNA synthesis in serum treated cells at low concentrations, cycloheximide also appears to prolong the time cells spend in DNA synthesis which could occur by

cycloheximide slowing down replication fork progression, or preventing maturation of short nascent DNA fragments into high molecular weight (trichloracetic acid precipitable) DNA. The observations that cycloheximide affects Ad 5 induced cellular DNA synthesis in a different way from serum induced DNA synthesis, and does not appear to substantially inhibit viral early protein synthesis, suggests that the control mechanisms for serum induced and Ad 5 induced DNA synthesis are different. Ad 5 infected cells do not show the cycloheximide induced delay in entry into DNA synthesis but generally show a lower level of incorporation of [^{3}H] thymidine. This suggests that low concentrations of the drug prevent synthesis of one or more proteins (presumably cellular) required to maintain cellular DNA replication but do not affect the initiation step in virus infected cells. This result provides confirmatory evidence for the suggestion in Chapter 3 (Section 3.3.6) that synthesis of a cellular protein is required continually to maintain cellular DNA replication induced by Ad 5 infection. The result also suggests that this protein is not required for entry into DNA synthesis (as control and drug treated cells appear to initiate DNA synthesis at the same time) and therefore implies that Ad 5 early proteins as well as other cellular proteins unaffected by these concentrations of cycloheximide are responsible for initiation.

6.3.6 CYCLOHEXIMIDE DELAYS AND PROLONGS G_2 + M AFTER SERUM STIMULATION

Results from previous sections with serum stimulated cells provided clear evidence that low concentrations of cycloheximide

delayed entry into S phase and also suggested that the drug was prolonging S phase. These experiments were mostly done by measurements of [^{3}H] thymidine incorporation into replicating DNA although one preliminary result was obtained using flow cytometry. This result showed essentially the same phenomenon as was obtained with [^{3}H] thymidine labeling of DNA (Table 6.2). In this section, the effects of cycloheximide on the kinetics of cellular proliferation is studied by measuring the proportion of cells in each phase of the cell cycle after drug treatment. This experiment was done with flow cytometry using the procedure described in Chapter 4.

Quiescent mouse cells were serum stimulated and then to some cultures cycloheximide was added at two different concentrations. Beginning at 22 h after serum addition and at 2 h intervals thereafter until 45 h cells were harvested and analysed by flow cytometry. The results of this experiment are tabulated as percentage of cells in each phase of the cell cycle (G_1, S, G_2 + M, or $>G_2$) after each treatment (Table 6.4), although one time point (22h after serum addition) is illustrated as flow cytometric determined DNA histograms in order to illustrate the marked effects on cell cycle kinetic parameters that cycloheximide has in mouse cells (Fig. 6.5).

Results in Fig. 6.5 and Table 6.4 showed that at the 22 h time-point mock infected cells contained almost 94% in G_1

FIGURE 6.5

DNA histograms of mouse cells stimulated to proliferate with serum, with and without cycloheximide. Quiescent mouse cells were treated with serum and then cycloheximide was added to some cultures at 0.02 µg/ml and 0.05 µg/ml. 22 h later these cells were harvested and stained for flow cytometric analysis of DNA content using EB and MMC. For details of staining procedure refer to Section 4.2.2. This experiment was done using the FACS IV in Canberra.

(A) Mock infected, G_1-arrested cells

(B) Serum stimulated control

(C) Serum stimulated plus 0.02 µg/ml cycloheximide

(D) Serum stimulated plus 0.05 µg/ml cycloheximide

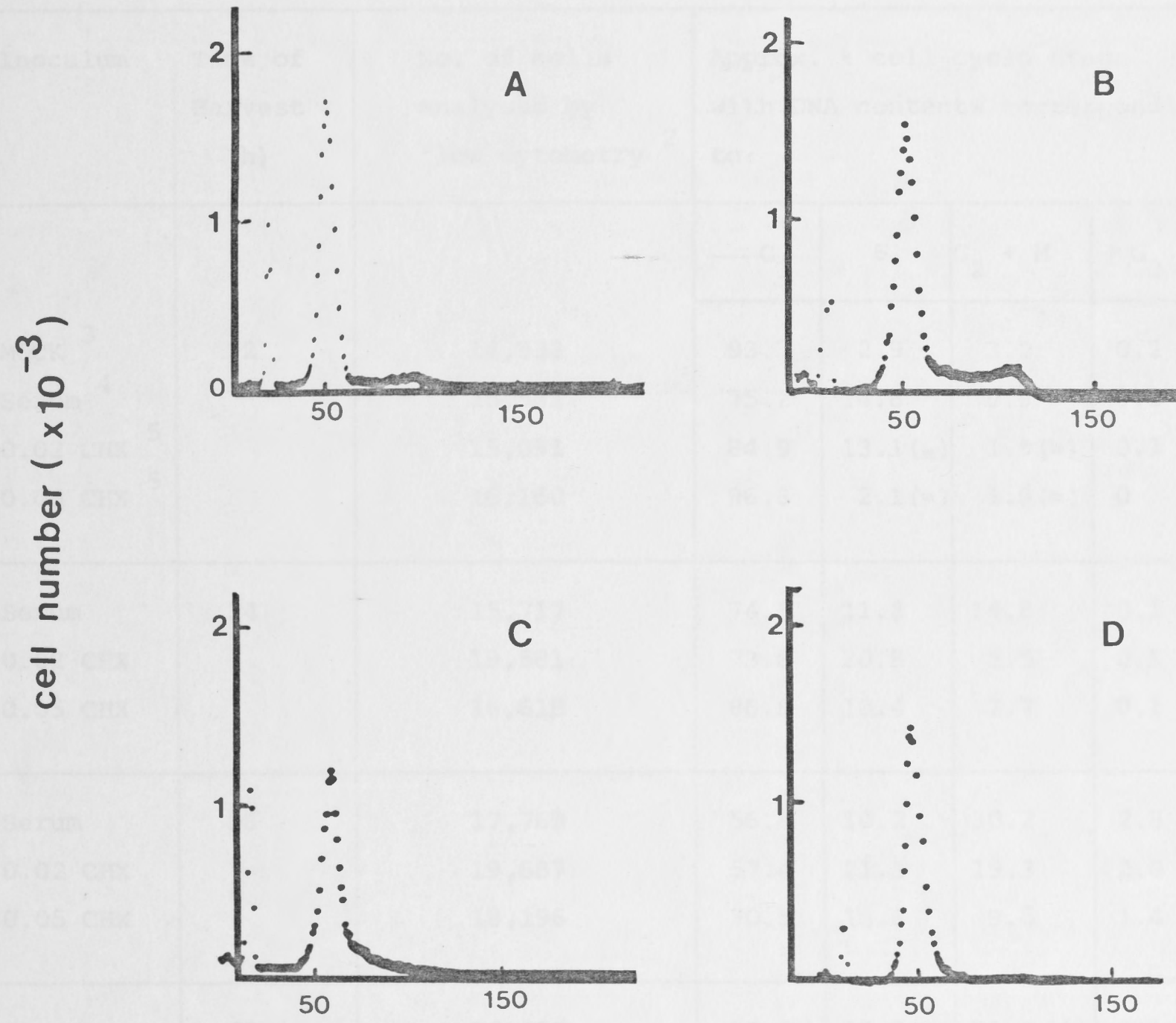

A
B
C
D
2
1
0
50
150
cell number (x10^{-3})
channel number

TABLE 6.4

Cell cycle analysis of serum stimulated, Cycloheximide treated mouse cells [1]

Inoculum	Time of Harvest (h)	No. of cells analysed by flow cytometry [2]	Approx. % cell cycle stage with DNA contents corresponding to:			
		—	G_1	S	G_2 + M	> G_2
MOCK [3]	22	14,932	93.7	2.9	3.2	0.2
Serum [4]		25,531	75.2	14.8	9.9	0.1
0.02 CHX [5]		15,091	84.9	13.1(≃)	1.9(≃)	0.1
0.05 CHX [5]		16,160	96.3	2.1(≃)	1.6(≃)	0
Serum	24	15,717	74.6	11.3	14.0	0.1
0.02 CHX		19,581	73.6	20.8	5.5	0.1
0.05 CHX		16,618	86.8	10.4	2.7	0.1
Serum	26	17,769	56.8	10.2	30.2	2.8
0.02 CHX		19,687	57.4	21.3	19.3	2.0
0.05 CHX		18,196	70.5	18.6	9.5	1.4
Serum	28	20,450	63.2	13.3	21.4	2.1
0.02 CHX		19,928	50.4	18.1	29.2	2.3
0.05 CHX		17,777	53.1	29.8	17.1	0
Serum	30	20,396	68.9	9.1	19.8	2.2
0.02 CHX		28,078	52.9	18.8	26.3	2.0
0.05 CHX		20,831	53.3	26.3	18.7	1.7
Serum	32	23,107	73.1	7.3	18.0	1.6
0.02 CHX		20,346	54.4	11.5	32.0	2.1
0.05 CHX		19,340	50.4	18.2	29.2	2.2

(continued over)

TABLE 6.4 (continued)

Inoculum	Time of Harvest (h)	No. of cells analysed by flow cytometry [2]	Approx. % cell cycle stage with DNA contents corresponding to:			
			G_1	S	G_2 + M	> G_2
MOCK [6]	35	19,149	85.0	4.3	9.5	1.2
Serum		20,016	73.3	9.5	15.8	1.4
0.02 CHX		19,250	66.9	9.0	21.7	2.4
0.05 CHX		21,086	55.0	13.4	29.2	2.4
Serum	37	19,641	71.6	9.3	17.3	1.8
0.02 CHX		19,452	64.7	9.6	23.8	1.9
0.05 CHX		20,434	50.1	12.7	34.6	2.6
Serum	39	24,582	73.1	10.1	15.2	1.6
0.02 CHX		19,523	70.2	7.2	20.5	2.1
0.05 CHX		18,845	57.3	10.0	30.0	2.7
Serum	41	19,505	72.9	6.3	18.3	2.5
0.02 CHX		30,130	70.0	7.6	20.7	1.7
0.05 CHX		19,793	60.5	10.0	27.3	2.2
Serum	43	27,086	77.0	7.9	14.7	0.4
0.02 CHX		25,416	72.6	5.5	19.8	2.1
0.05 CHX		19,607	64.9	7.0	26.0	2.1
Serum	45	19,262	78.6	4.7	15.0	1.7
0.02 CHX		18,308	76.6	7.4	14.0	2.0
0.05 CHX		18,396	70.7	7.1	19.9	2.3

(continued over)

TABLE 6.4 (continued)

1 Cells were arrested and serum stimulated as described in Chapter 2

2 Flow cytometry using the FACS IV was done as described in the legend to Fig. 6.5

3 MOCK = G_1-arrested (0.2% BS)

4 Serum = serum stimulated (10% FCS)

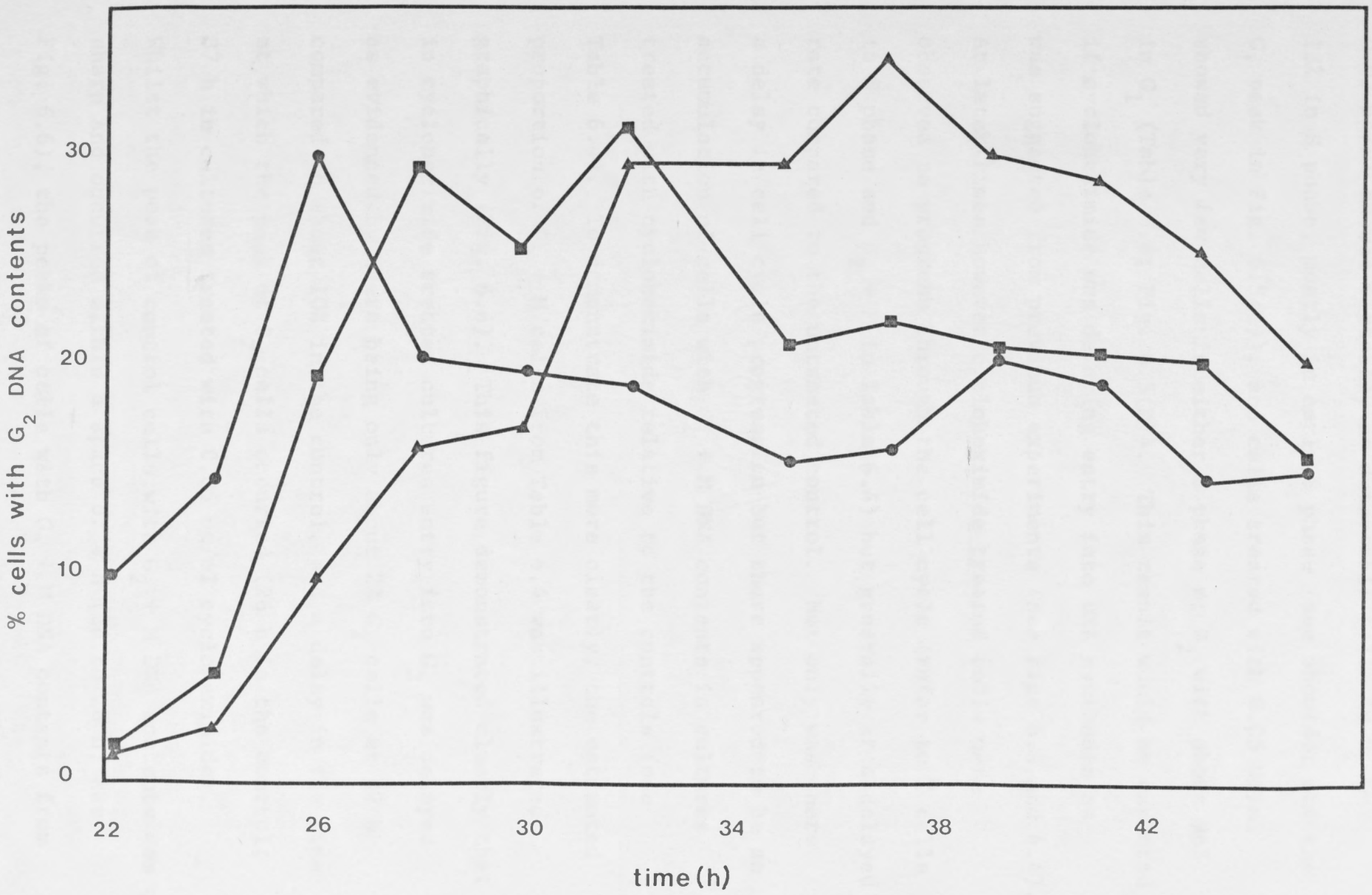
5 0.02, 0.05 CHX = serum stimulated plus 0.02 or 0.05 µg/ml cycloheximide

6 This experiment was done in two parts, staggering the serum addition. Samples beginning at 35 h were serum stimulated 13 h before the samples assayed 22 to 32 h

FIGURE 6.6

Proportion of cells with G_2 DNA contents after treatment with cycloheximide taken from Table 6.4. Quiescent mouse cells were treated with serum and then cycloheximide was added to some cultures at 0.02 µg/ml and 0.05 µg/ml. At indicated times cells were harvested and stained for flow cytometric analysis. This experiment was done using the FACS IV in Canberra.

Symbols:
- ● untreated serum control
- ■ serum plus 0.02 µg/ml cycloheximide
- ▲ serum plus 0.05 µg/ml cycloheximide


% cells with G_2 DNA contents
30
20
10
0
22
26
30
34
38
42
time (h)

compared to 75% in G_1 in serum stimulated cells, with the remainder in S phase or G_2 (see Table 6.4). Cells treated with 0.02 µg/ml showed virtually no detectable cells in G_2 but 13% in S phase, mostly in early S phase (see shoulder off the G_1 peak in Fig. 6.5(c)), and cells treated with 0.05 µg/ml showed very few cells in either S phase or G_2 with about 96% in G_1 (Table 6.4; Fig. 6.5(D)). This result would be expected if cycloheximide was delaying entry into DNA synthesis as was suggested from previous experiments (see Figs 6.3 and 6.4). At later times however cycloheximide treated cells were observed to progress through the cell cycle (refer to % cells in S phase and G_2 + M in Table 6.4) but generally at a delayed rate compared to the untreated control. Not only was there a delay in cell cycle progression but there appeared to be an accumulation of cells with G_2 + M DNA contents in cultures treated with cycloheximide relative to the controls (see Table 6.4). To demonstrate this more clearly, the estimated proportion of G_2 + M cells from Table 6.4 was illustrated graphically (Fig. 6.6). This figure demonstrated clearly that in cycloheximide treated cultures entry into G_2 was delayed as evidenced by there being only about 2% G_2 cells at 22 h compared to about 10% in the control, and a delay in the time at which the peak of G_2 cells occurred (26 h in the control; 37 h in cultures treated with 0.05 µg/ml cycloheximide). Whilst the peak of control cells with G_2 + M DNA contents was quite sharp and occurred within a space of 4 h (24 to 28 h; see Fig. 6.6), the peaks of cells with G_2 + M DNA contents from cycloheximide treated cultures were very broad. Thus the time taken for drug treated cells to progress from the end of S phase back to G_1 was also prolonged.

Delayed entry into G_2 seems most likely to be due to the delayed G_1 to S phase transition and prolongation of S phase shown previously. However, it is possible that there is some specific protein required to be synthesised to threshold levels in order to effect the S to G_2 phase transition. Unfortunately, there is no obvious way to distinguish between cells blocked at the S/G_2 border or blocked somewhere between the end of S phase and cytokinesis.

Similarly, slowed progression through the G_2 + M phases of the cell cycle might also be as a result of a general lengthening of G_2, M or both, or reduced synthesis of a specific protein required for cells to traverse a restriction point, for instance between G_2 and mitosis. Certainly, synthesis of the chromosome condensation factor, required for metaphase condensation (Prescott, 1976) is probably delayed. Inhibition of this factor would delay exit from G_2+ M and therefore prolong the time cells contained a 4 n amount of DNA.

To see if cycloheximide was prolonging the G_2 + M phase of the cell cycle directly another experiment was done. In this experiment cycloheximide was added at the time of serum stimulation or delayed 22 h. Cells were then harvested at indicated times beginning 23 h after serum addition. The results of this experiment are also tabulated as cell cycle kinetic parameters (Table 6.5) and the proportions of cells in the G_2 + M phases of the cell cycle are also illustrated graphically (Fig. 6.7) as was done in Fig. 6.6.

TABLE 6.5

Effect of Cycloheximide on cell cycle progression when added with Serum or delayed 22 h [a]

Inoculum	Time of Cycloheximide [b] Addition (h)	Time of assay (h)	Approx. % cell cycle stage of cells with DNA contents [c] corresponding to:			
			G_1	S	G_2 + M	$>G_2$
MOCK [d]	-	23	78.7	4.4	14.1	2.8
Serum [e]	-	"	56.9	9.8	29.1	4.2
0.05 CHX [f]	0	"	69.1	27.5	1.0 [h] (≃)	2.4
"	22	"	50.5	10.6	35.4	3.6
Serum	-	24	56.7	10.0	29.2	4.0
0.05 CHX	0	"	64.2	23.4	10.4	2.1
"	22	"	47.8	11.5	36.3	4.7
Serum	-	26	62.2	9.2	25.8	2.8
0.05 CHX	0	"	67.2	17.4	13.0	2.5
"	22	"	52.0	10.7	33.5	4.0
Serum	-	28	64.6	9.4	23.3	2.7
0.05 CHX	0	"	54.1	20.5	22.1	3.2
"	22	"	60.3	9.4	26.9	3.4
Serum	-	30	68.9	10.8	18.3	1.9
0.05 CHX	0	"	55.8	18.8	22.8	2.6
"	22	"	66.1	7.8	24.8	2.3
Serum	-	32	68.0	10.0	19.9	2.2
0.05 CHX	0	"	49.7	15.3	31.0	3.8
"	22	"	74.2	6.8	16.1	2.0

(continued over)

TABLE 6.5 (continued)

Inoculum	Time of Cycloheximide Addition (h) [b]	Time of assay (h)	Approx. % cell cycle stage of cells with DNA contents corresponding to: [c]			
			G_1	S	G_2 + M	> G_2
MOCK [g]	-	36	84.4	4.0	9.8	1.7
Serum	-	"	63.5	10.2	23.6	2.7
0.05 CHX	0	"	54.7	16.4	26.4	2.8
"	22	"	71.7	10.1	15.7	2.5
Serum	-	37.5	58.9	12.2	24.8	4.1
0.05 CHX	0	"	55.4	11.7	30.0	3.0
"	22	"	69.9	11.8	15.8	2.4
Serum	-	39	61.5	9.9	25.3	3.3
0.05 CHX	-	"	59.6	9.7	27.7	2.6
"	22	"	70.8	10.3	15.3	3.1
Serum	-	41	63.8	8.5	25.4	2.3
0.05 CHX	0	"	55.2	10.8	31.4	2.6
"	22	"	68.7	11.6	16.7	2.6
Serum	-	43	66.5	9.1	21.8	2.6
0.05 CHX	0	"	57.8	8.5	30.1	3.1
"	22	"	65.6	10.2	21.2	3.0
Serum	-	60	79.7	5.3	20.3	1.5
0.05 CHX	0	"	69.0	11.1	18.2	1.8
"	22	"	74.1	7.2	17.2	1.4

(continued over)

TABLE 6.5 (continued)

a Mouse cells were made quiescent by culturing for 2 days in 0.2% BS then stimulated into a cell cycle by addition of 10% FCS

b The concentration of cycloheximide used in this experiment was 0.05 µg/ml

c DNA contents were analysed by flow cytometry using the procedure described in Section 4.2.2. This experiment was carried-out using the FACS IV in Canberra

d MOCK represents G_1-arrested cultures

e Serum represents serum stimulated controls (no cycloheximide)

f 0.05 CHX represents serum stimulated controls treated with 0.05 µg/ml cycloheximide

g This experiment was done in two parts, staggering the serum addition. Samples beginning at 36 h were serum stimulated 14 h before the samples assayed from 23 to 32 h

h There were very few cells detectable in the G_2 phase of the cell cycle at this time. This figure is an approximate guess.

FIGURE 6.7

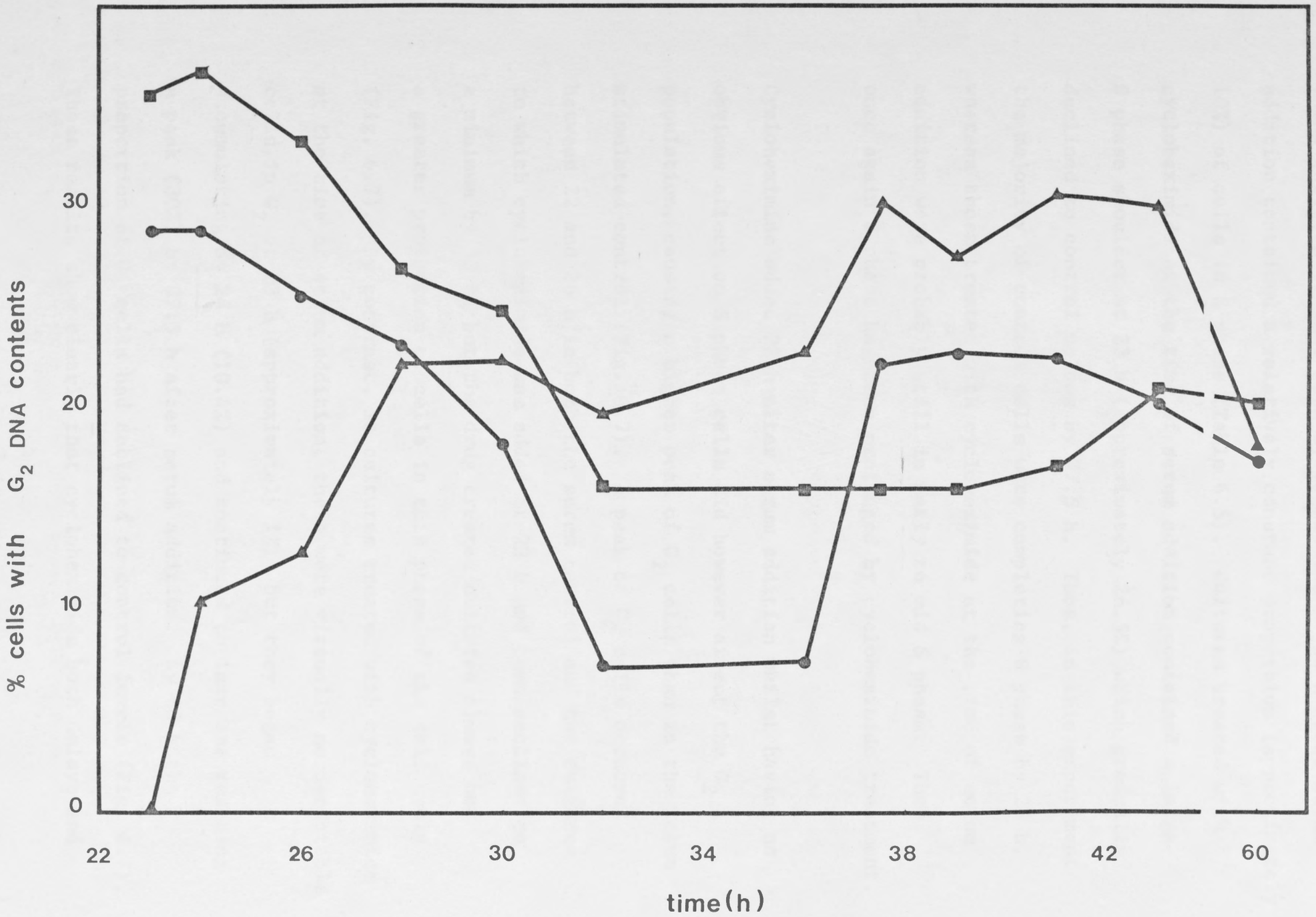
Proportion of mouse cells with G_2 DNA contents after cycloheximide treatment. The data illustrated in this figure were taken from that in Table 6.5. Quiescent mouse cells were serum stimulated and cycloheximide was added immediately or delayed 22 h. Beginning at 23 h and at indicated times thereafter cells were harvested and stained for analysis by flow cytometry. For details of the staining procedure refer to Section 4.2.2. This experiment was done using the FACS IV in Canberra.

Symbols:

● serum stimulated control (no cycloheximide)

■ serum stimulated, 0.05 µg/ml
cycloheximide added with serum

▲ serum stimulated, 0.05 µg/ml
cycloheximide added 22 h after serum


% cells with G2 DNA contents
30
20
10
0
22
26
30
34
38
42
60
time(h)

Over the time course of the experiment the serum controls and those cultures treated with cycloheximide at 22 h after serum addition contained a relatively constant proportion (approximately 10%) of cells in S phase (Table 6.5). Cultures treated with cycloheximide at the time of serum addition contained a large S phase shoulder at 23 h (approximately 26.5%) which gradually declined to control values by 37.5 h. Thus, in this experiment the majority of control cells were completing S phase by 23 h, whereas those treated with cycloheximide at the time of serum addition were probably still in early to mid S phase. Thus once again S phase has been prolonged by cycloheximide treatment.

Cycloheximide added 22 h after serum addition whilst having no obvious effect on S phase cells did however affect the G_2 population, causing a higher peak of G_2 cells than in the serum stimulated control (Fig. 6.7). A peak of G_2 cells occurred between 22 and 26 h in both the serum control and the cultures to which cycloheximide was added at 22 h and then declined to a minimum by 32 h, but the drug treated cultures always had a greater proportion of cells in this phase of the cell cycle (Fig. 6.7). By contrast, in cultures treated with cycloheximide at the time of serum addition, there were virtually no detectable cells in G_2 at 23 h (approximately 1%), but they began accumulating by 24 h (10.4%) and continued to increase reaching a peak (30%) by 37.5 h after serum addition. By 60 h the proportion of G_2 cells had declined to control levels (Fig. 6.7). These results show clearly that cycloheximide both delays and

prolongs the combined G_2 + M phases (4n DNA content) of the cell cycle, as there is both a slowed rate of accumulation of 4n cells (cycloheximide added at the time of serum addition), and also a slowed decrease in cells with 4n DNA contents compared to controls (cycloheximide added 22 h after serum). Thus, cycloheximide delays exit from the G_2 + M phases of the cell cycle.

This experiment showing delayed progression through the G_2 + M phases of the cell cycle when cycloheximide is added 22 h after serum confirms that there is a second delay and suggests that there are at least two functionally dependent regulatory points of cellular proliferation: one check-point regulates entry into DNA synthesis (G_1 to S phase) and there is at least one other which regulates traverse of the G_2 + M phases to cytokinesis.

6.4 DISCUSSION

The results of experiments reported in this chapter have provided evidence that low concentrations of cycloheximide (which inhibit protein synthesis up to 50% of untreated controls) cause a delay in entry into S phase in cells stimulated to proliferate with serum growth factors. This delay results from a specific increase in the time cells spend in the G_1 phase of the cell cycle, which was shown both by [^{3}H] thymidine labeling of replicating DNA and by flow cytometric analysis. Other experiments provided evidence that cycloheximide also delays progression through S phase (see Fig. 6.4), and through the G_2 + M phases of the cell cycle (Figs. 6.6 and 6.7). The observation that 0.05 µg/ml cycloheximide causes a delay in exit from G_2 within 1 to 5 h of addition (see Fig. 6.7) suggests that the drug is probably inhibiting synthesis of one or more proteins that are rapidly metabolised, as proteins with long half-lives (at least >5 h) would not be affected in this short time period (Berlin and Schimke, 1965). Thus, traverse of the G_2 + M (4n) phases of the cell cycle to cytokinesis might be partly dependent on the synthesis of labile regulatory proteins, one of which might be the chromosome condensation factor mentioned above, although very little is known about this factor (see Prescott, 1976).

The delay in entry into DNA synthesis might also be partly due to inhibition of synthesis of labile initiator proteins (Schneiderman et al., 1971; Rossow et al., 1979), but the long drug treatments used in the experiments reported in this chapter

make it impossible to distinguish between this interpretation and one which argues in favor of the synthesis of several proteins in G_1 being inhibited which delays the G_1 to S phase transition. The observation in Fig. 6.4 that delayed entry into S phase occurs within 12 h of cycloheximide addition does however imply that proteins with half lives of <12 h would be preferentially affected and therefore suggests these might be responsible for effecting the G_1 to S phase transition. This would still include several different classes of G_1 specific proteins such as ornithine decarboxylase (Cheetham and Bellett, 1982).

The most economical general conclusion from these results is that low concentrations of cycloheximide delay cell cycle progression at a minimum of three (G_1, S and G_2 + M) different points in the rodent cell cycle. This would occur by cycloheximide interfering with synthesis of several classes of proteins (some of which might be labile regulatory proteins) required continually for normal cell cycle progression.

In adenovirus infected rodent cells a different situation would appear to exist. Cycloheximide (up to 0.05 µg/ml) does not appear to inhibit entry into DNA synthesis but does reduce the total amount of [^{3}H]thymidine incorporated into DNA in a dose dependent manner (see Figs. 6.1, 6.2, and 6.4). Since adenovirus early proteins are apparently not inhibited by these low concentrations of cycloheximide, the results suggest that Ad 5 is inducing cellular DNA replication in a way which is different from serum growth factors. Since cycloheximide has little or no

effect on entry into DNA synthesis in Ad 5 infected cells, but rather on the rate of DNA replication, cycloheximide is probably inhibiting synthesis of one or more cellular proteins which are required to maintain DNA replication as was suggested from the results presented in Section 3.3.6. That is, early viral proteins and cycloheximide sensitive cellular proteins are probably required to maintain cellular DNA replication induced by Ad 5, but viral proteins and cycloheximide insensitive cellular proteins are required for initiation. Thus the controls of adenovirus induced cellular DNA replication are different from the controls of normal serum induced DNA replication. These results therefore support and extend the observations reported in Chapter 5 that Ad 5 can induce cellular and viral DNA replication under conditions when normal cellular proliferation is prevented.

CHAPTER 7

GENERAL DISCUSSION

7.1 INTRODUCTION

The studies discussed in this thesis have examined the effects of adenovirus type 5 (Ad 5) and several mutants which show altered or defective transforming properties, on various aspects of the mammalian cell cycle. The intentions were to (1) attempt to determine the region of the adenovirus genome responsible for causing altered cell cycling; (2) investigate the relationship between cell cycle alterations and adenovirus induced transformation; and (3) hopefully increase understanding of the possible mechanisms by which DNA tumor viruses cause transformation of cells and increase the risk of tumorogenesis. The pertinent findings of this thesis will now be discussed in relation to these aims, with suggestions for further research.

7.2. THE GENETICS OF INDUCTION OF CELLULAR DNA REPLICATION BY ADENOVIRUS

Results of experiments presented in Chapter 3 provided evidence that the most likely region of the Ad 5 genome responsible for inducing cellular DNA replication is early region 1A (0-4.5% in map units). However, as this region also regulates expression of other early region transcriptional units (Lewis et al., 1979), it was not possible to unequivocally distinguish between a direct requirement for early region 1A and an indirect requirement due to the dependence of early region 4 on 1A expression. Early regions 1B, 2A, and 2B appeared not to be responsible for induction based on experiments with other mutants, and early region 3 was shown to be nonessential in hamster cells by Rossini et al.,(1981).(These findings were discussed in detail in Section 3.4.)

Experiments presented in Chapter 4, in which total cell cycle analysis was done, extended the observations made in Chapter 3. Ad 5 wild-type and all mutants excepting one deleted in early region 1A (d1 312) caused marked cell cycle alterations. Not only was the proportion of cells undergoing cellular DNA replication increased by virus infection, but so too was the proportion of cells in the G_2 phase as well as an accumulation of cells having aneuploid and polyploid DNA contents (this often involved as many as 1/3 of the total cell population). As was observed in Chapter 3, mutants defective in early regions 1B, 2A and 2B functions were not defective for causing cell cycle alterations, whereas the mutant deleted in early region 1A had no effect on the rodent cell cycle. These results once again suggested that the region of the Ad 5 genome responsible for causing the formation of cells with aneuploid and polyploid DNA contents is early region 1A. However, until early region 4 is excluded as a candidate, this conclusion cannot be considered definite.

7.3 RELATIONSHIP OF ADENOVIRUS TRANSFORMATION TO THE INDUCTION OF CELL CYCLE ABERRATIONS

The smallest DNA fragments with transforming activity are the left hand 6.8% of Ad 12 DNA (Shiroki et al., 1977); the left hand 7.3% of Ad 2 or Ad 5 DNA (Graham et al., 1977; van der Eb and Houweling, 1977); and the left hand 8.1% of Ad 7 DNA (Sekikawa et al., 1978). These DNA fragments have the capacity to induce complete transformation in the same way as whole viral DNA. Recently smaller fragments, such as the left hand

4.5% of Ad 5 DNA (Houweling et al., 1980) have been shown to transform cells incompletely or partially. These results implied that early regions 1A and 1B were the minimum essential requirements for complete transformation by adenovirus, but partial transformants could be isolated after transfection with only early region 1A. More recently still, mutants deleted in either early regions 1A or 1B (d1 312 and d1 313 which were studied in this thesis), were tested for transforming ability in a continuous line of rat cells (Shiroki et al., 1981). These workers found that partial transformants could be isolated after d1 313 infection (deleted in early region 1B), but no transformants could be isolated after d1 312 infection (deleted in early region 1A). Transformants isolated after wild-type Ad 5 infection could be cloned in soft agar and continuous cell lines established, but this could not be demonstrated for transformants isolated after d1 313 infection. These results suggested that early region 1A was an essential initiating region for transformation, and probably early region 1B was required for stabilising the transformed phenotype. The importance of early region 1A suggested by these results was in agreement with results obtained by Houweling et al., (1980) (see above). Indeed the phenotypes of the partial transformants isolated after d1 313 infection (Shiroki et al, 1981) were similar to those isolated after transfection with a DNA fragment containing only early region 1A (Houweling et al., 1980).

The situation however is complicated by reports that d1 313 did not transform primary rat embryo or rat embryo brain cells

(Jones and Shenk, 1979a), and that a host-range defective mutant in early region 1A (hr 1) (Harrison et al., 1977) induced partial transformants in baby rat kidney cells, but not in rat embryo or rat embryo brain cells (Graham et al., 1978). Such results suggested that the physiology of the cells plays a major determining role in transformation. These studies and others were discussed in more detail in Chapter 4.

The above studies on the genetics of adenovirus transformation and the studies reported in Chapters 3 and 4 on the genetics of adenovirus induced cell cycle alterations, have provided evidence that the region of the Ad 5 genome responsible for causing transformation is the same region that is responsible for causing cell cycle alterations; that is, early region 1A. Early region 1A encodes a series of spliced mRNAs which can be translated in vitro into 4 (Lewis et al., 1979) or 2 (Ricciardi et al., 1981) polypeptides (see Chapter 4). Thus whether some or all of the polypeptides required to initiate a round or rounds of cellular DNA replication are the same as those required to initiate transformation of cells still requires considerable clarification. The fact that both processes are regulated by the same transcriptional region of the virus genome would however seem to suggest that the two processes are related. Induction of an aberrant cell cycle, resulting in cells with abnormal DNA contents, may in some cases (although not all) lead to subsequent transformation.

Alterations to the cell cycle resulting in cells with abnormal DNA contents are however not the sole prerequisites for induction of transformation because (1) polyploids occur at much higher frequency than the transforming event; (2) not all transformed and tumor cells have altered DNA contents (Gallimore and Paraskeva, 1979); and (3) the nature of the cell appears to play a role in the initiation of transformation. However, alteration of cell cycle controls by adenovirus may contribute in some cases to the transformed phenotype.

Ad 5 mutants containing defects in regions other than early regions 1A and 1B, have also been shown to affect transformation frequency. For example, ts 125 causes a higher than wild-type frequency of transformation at both permissive and nonpermissive temperatures in rat cells (Ginsberg et al., 1974). In addition, early region 2A regulates expression of early region 4 in wild-type Ad 5 which is impaired in ts 125 infected cells incubated at the nonpermissive temperature (Nevins and Winkler, 1980). These observations with mutant ts 125, and those discussed above serve to emphasise that the induction of transformation by adenovirus is not simply explained, as many factors appear to play a role in regulating the initiation of transformation.

7.4 ALTERED CONTROLS OF CELLULAR PROLIFERATION, ADENOVIRUS INFECTION, AND TRANSFORMATION

A commonly observed characteristic of virally transformed cells in vitro is their inability to grow under conditions which would prevent normal cellular proliferation. Much of the evidence in the

literature for this was reviewed in Sections 1.3.2 and 3.1, 5.1 and 6.1 of the experimental chapters. Experimental results reported in Chapters 5 and 6 provided evidence that Ad 5 infection of rodent cells modifies a number of regulatory controls of normal cellular proliferation, including cyclic AMP and Ca^{2+} sensitive control points (Chapter 5). Ad 5 infected cells also respond differently to partial inhibition of protein synthesis with cycloheximide. Whereas normal cells showed delayed entry into DNA synthesis after cycloheximide treatment Ad 5 infected cells did not. For more details of these adenovirus induced modifications refer to experiments in Chapters 5 and 6.

Experiments showing that Ad 5 could (1) maintain cellular DNA replication when Ca^{2+} ions were reduced below physiological levels or cyclic AMP raised above physiological levels (Chapter 5); (2) induce cellular DNA replication in the total absence of serum growth factors (Fig. 3.7); and (3) induce cellular DNA replication in brain cells of baby rats infected in vivo (Fig. 3.8), have provided some clues as to the way Ad 5 might induce transformation; particularly as adenovirus transformants are often isolated from rat brain cultures (Gallimore, 1974) and in medium containing a low Ca^{2+} concentration (Freeman et al., 1967). In order for a productive cycle of adenovirus replication to occur, the virus may need to force cells into a cell cycle, thereby stimulating a number of cellular activities required for virus growth and viral DNA replication. This would occur in both a permissive and a nonpermissive infection, but in the latter case, in some cells multiple rounds of cellular

DNA replication would result leading to the formation of cells with aneuploid and polyploid DNA contents. The ability to stimulate a cell cycle under many restrictive conditions would be an evolutionary adaptation allowing adenovirus to capitalise on host cell machinery as efficiently as possible for a productive virus infection to occur. In a semipermissive infection, fewer virus particles are produced, there is some but less cell death, and cells with altered DNA contents form. Permanent alteration of cell growth cycle responses to environmental signals could result from integration and controlled expression of early region 1A genes, genetic damage and imbalance caused by uncontrolled early region 1A expression during the first few days after infection, or both. Under conditions which restrict growth of normal cells, some infected cells that have altered growth properties might be preferentially selected, and subsequently develop into transformed cells.

7.5 GROWTH GENES AND TRANSFORMATION

Many of the studies reviewed in previous chapters, as well as the experimental data reported in this thesis, have emphasised the marked and diverse effects adenovirus has on the mammalian cell growth cycle. The argument that these modifications to the cell cycle are important early events which may lead to transformation of cells and tumorogenesis has been stressed in this chapter, and throughout the thesis. It is interesting in this context, that many well-characterised transforming viruses cause cells to undergo at least one round of cellular DNA replication. Aside from the adenoviruses and SV 40 (see

references cited in Chapter 4), polyomavirus (Dulbecco et al., 1965), Rous Sarcoma virus (Kobayashi and Kaji, 1978), human cytomegalovirus (Gönczöl et al., 1978) and Epstein-Barr virus (Gerber and Hoyer, 1971) have all been shown to induce cellular DNA replication. For SV 40, one of the genes associated with transformation (gene A, coding for T protein) has been clearly shown to be responsible for inducing cellular DNA replication (see references in Section 3.1), as has the transforming (src) gene of Rous sarcoma virus (Kobayashi and Kaji, 1978). Furthermore, experimental results from this thesis have provided evidence that adenovirus genes that affect cell growth cycle regulation are associated with the transforming region (see Chapters 3 and 4).

The concept of "growth genes" being modified during oncogenic transformation of cells is not a new one (see review by Chan, 1981) but it is an important one, as it may be one of the few common denominators in cancer causality, not just in virus induced cancers. For example, in the platyfish, *Platypoecilus maculatus*, there is a set of genes called 'Tu' which code for the oncogenic phenotype (Anders and Anders, 1978), but in the nontumorogenic state they may play a role in developmental and regenerative processes. In the normal differentiated state these genes are repressed, but when the repressor region (R genes; Anders and Anders, 1978) is mutated by chemical treatment, one of the Tu genes is expressed and whole body melanomas develop. Thus, in this system too, transformation and cellular growth modifications are linked. If there is a single cause of cancer,

irrespective of the initiating agent, then as Todaro (1980) argues a good candidate would seem to be "growth gene" alterations. The results of experiments reported in this thesis are consistent with such an hypothesis.

7.6 FUTURE EXPERIMENTS

In order to investigate in more detail the relationship between adenovirus induced cell cycle alterations and transformation, there would seem to be two major directions to take. The first would be to generate deletion mutants of Ad 5 in early region 4 and test these for the ability to generate cell cycle alterations. In this way unequivocal evidence would be obtained that adenovirus early region 1A is the coding sequence for polypeptides responsible for cell growth cycle alterations. Deletions in Ad 5 early region 4 could be generated using the BAL-31 nuclease (Legerski et al., 1978) and the approach outlined in Section 3.4. The difficulty would be in generating a permissive cell line for such deletion mutants. This might be achieved by linking an endonuclease fragment containing early region 4 to the herpesvirus thymidine kinase gene, then cotransducing this fragment into thymidine kinase defective cells and selecting for cells expressing the thymidine kinase gene. One could then test for the unselected marker (early region 4) by attempting to grow the mutants deleted in early region 4.

Another but related approach would be to purify in vitro translated polypeptides encoded by early region 1A (see Lewis et al., 1979; Ricciardi et al., 1981) and microinject them into arrested cells using the techniques described by

Graessman et al.,(1980). These approaches would enable the determination of not only the coding region, but also the exact polypeptide responsible for causing alterations to the mammalian cell cycle.

In order to see whether cells with altered DNA contents might contribute to transformation and tumorogenicity another approach could be adopted. Using the sorting function on the FACS IV, cells with aneuploid and polyploid DNA contents could be separated from cells having the normal G_1 and G_2 diploid DNA contents. Such separated cell populations could then be independently tested for transformation frequency in vitro, and tumorogenicity in appropriate animals. Preliminary experiments have indicated that this approach is feasible although not without its problems. Separation of cells requires vital staining with a membrane permeable bisbenzimide (Hoechst Dye No. 33342) that has little effect on the mammalian cell cycle, followed by sterile separation and then reculturing. All of these steps have some difficulties associated with them.

7.7 FINALE

Experiments reported in this thesis have provided some evidence indicating which adenovirus genes are responsible for altering cell cycle growth controls, and some effects on the cell cycle of expression of these genes have been described. These results demonstrating altered cell cycle controls by adenovirus infection go part way towards an explanation of how this virus causes cellular transformation, thus increasing the risk of tumorogenesis. However, clearly more experimental work needs to be done to investigate cell cycle alterations and its relationship to transformation. Some possibilities for further investigation have been suggested.

Once again, after all the "exploring" we have "arrived where we started", but rather than knowing "it for the first time" perhaps there is a glimmer of recognition.

APPENDICES

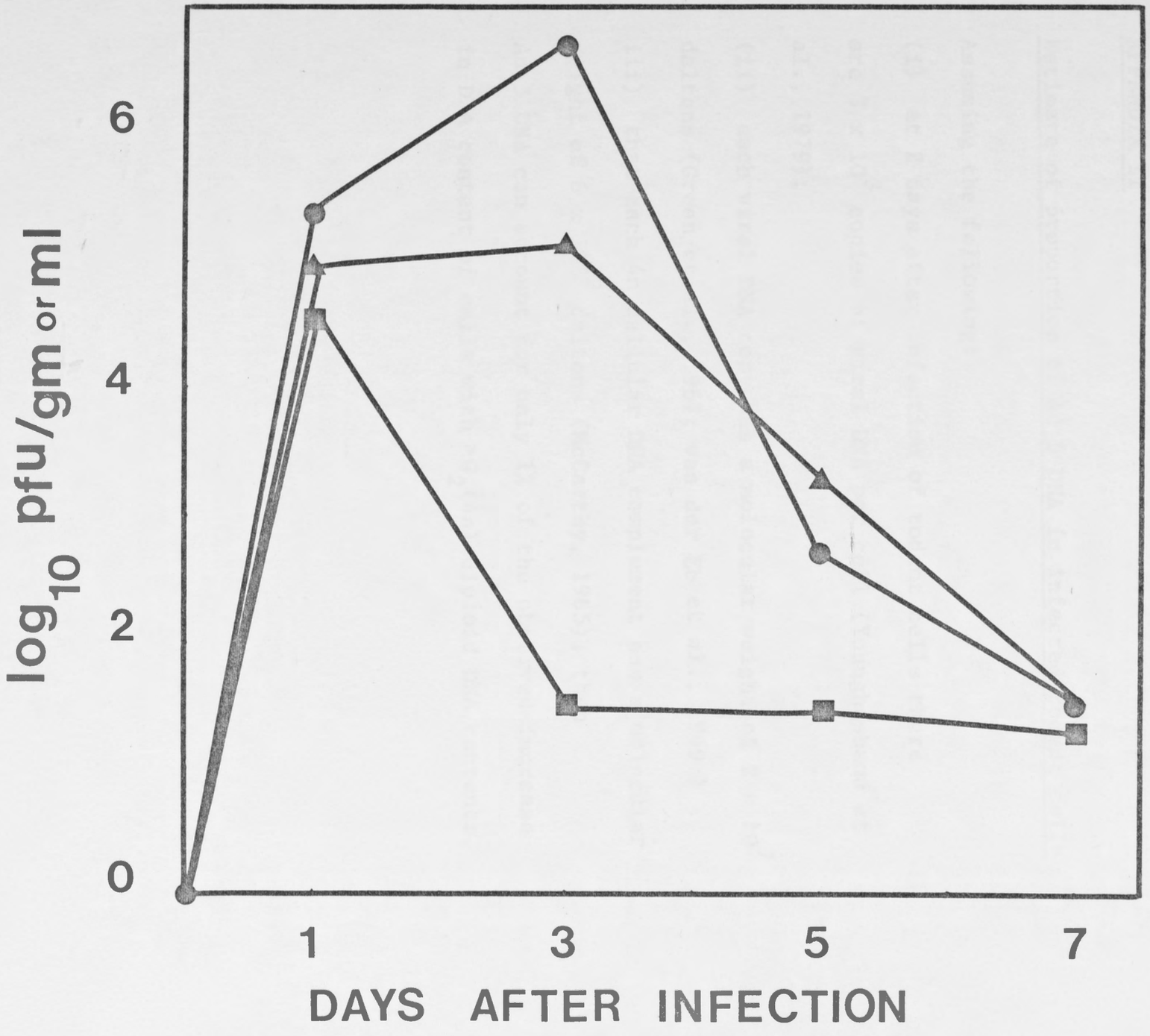
APPENDIX I

SFV replication in rat brains. Groups of rats were infected with 10^6 plaque forming units (pfu) intraperitoneally in 0.1 ml or intracerebrally with 3×10^5 pfu SFV in 30 µl. At indicated times 4 rats were sacrificed from each group and blood and brains were removed and frozen as described in Section 2.1.5. Virus titrations were done according to the method of Berger (1980).

Symbols: ● intracerebral inoculation; from brain tissue

▲ intraperitoneal inoculation; from brain tissue

■ " " ; from serum


6
4
2
0
log10 pfu/gm or ml
1
3
5
7
DAYS AFTER INFECTION

APPENDIX II

Estimate of proportion of Ad 5 DNA in infected rodent cells

Assuming the following:

(i) at 2 days after infection of rodent cells there are 3×10^3 copies of viral DNA per cell (Younghusband et al., 1979);

(ii) each viral DNA copy has a molecular weight of 2×10^7 daltons (Green et al., 1967; van der Eb et al., 1969)

iii) that each 4n cellular DNA complement has a molecular weight of 6×10^{12} daltons (McCarthy, 1965); then

Ad 5 DNA can account for only 1% of the observed increase in DNA content of cells with $>G_2(4n)$ diploid DNA contents.

APPENDIX III

Expression of the Ad 5 DNA-binding protein in Ad 5 and ts 125 infected rat cells [a] at permissive (32.5°C) and nonpermissive (39.5°C) temperatures for mutant replication

Inoculum	Temp (°C)	% cells expressing 'P' antigens [b,c]
MOCK	32.5	0
Ad 5	"	47.6
ts 125	"	36.3
MOCK	39.5	0
Ad 5	"	30.0
ts 125	"	2.4

a Quiescent rat cells were infected as described in Section 2.1.4 and all cells were harvested for antibody staining 3 days after infection.

b Cells were harvested and stained with P antiserum as described in Section 2.2.4. Between 200 and 300 cells were counted for each treatment.

c P antiserum reacts primarily with the Ad 5 DNA-binding protein, but also to a small extent with the Ad 5 T antigens (Russell et al., 1967).

Conclusion:

Ts 125 is defective for synthesis of P antigens (i.e. most likely the DNA-binding protein) at 39.5^{o}C which suggests that this mutant is indeed ts 125 and not another mutant or revertant. This result supports my conclusions in Section 3.4.

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